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Using Natural Products to Treat Resistant and Persistent Bacterial Infections

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USING NATURAL PRODUCTS TO TREAT RESISTANT AND PERSISTENT BACTERIAL
INFECTIONS

BY

ROBERT W DEERING

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PHARMACEUTICAL SCIENCES

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OF

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ABSTRACT

Antimicrobial resistance is a growing threat to human health both worldwide and in the United States. Most concerning is the emergence of multi-drug resistant (MDR) bacterial pathogens, especially the 'ESKAPE' pathogens for which treatment options are dwindling. To complicate the problem, approvals of antibiotic drugs are extremely low and many research and development efforts in the pharmaceutical industry have ceased, leaving little certainty that critical new antibiotics are nearing the clinic. New antibiotics are needed to continue treating these evolving infections. In addition to antibiotics, approaches that aim to inhibit or prevent antimicrobial resistance could be useful. Also, studies that improve our understanding of bacterial pathophysiology could lead to new therapies for infectious disease. Natural products, especially those from the microbial world, have been invaluable as resources for new antibacterial compounds and as insights into bacterial physiology. The goal of this dissertation is to find new ways to treat resistant bacterial infections and learn more about the pathophysiology of these bacteria. Investigations of natural products to find molecules able to be used as new antibiotics or to modulate resistance and other parts of bacterial physiology are crucial aspects of the included studies.

The first included study, which is reported in chapter two, details a chemical investigation of a marine *Pseudoalteromonas* sp. Purification efforts of the microbial metabolites were guided by testing against a resistance nodulation of cell division model of efflux pumps expressed in *E. coli*. These pumps play an important role in the resistance of MDR Gram negative pathogens such as *Pseudomonas aeruginosa* and

Enterobacteriaceae. Through this process, 3,4-dibromopyrrole-2,5-dione was identified as a potent inhibitor of the RND efflux pumps and showed synergistic effects against the *E. coli* strain with common antibiotics including fluoroquinolones, beta-lactams, tetracyclines, aminoglycosides, and chloramphenicol. The efflux pump inhibitory mechanism was further proved through an accumulation assay with the Hoechst dye 33342.

In chapter three, we report the discovery of a 1,2-benzisoxazole with new antibacterial activity against MDR *A. baumannii*, a pathogen with a critical need of new treatments. This compound was produced by bacterial fermentation and synthetic preparation and shows minimum inhibitory concentrations as low as 6.25 µg/mL against a panel of four clinically relevant *A. baumannii* strains. Key structure activity relationships were demonstrated using synthetic analogs of the lead 1,2-benzisoxazole. We advocate for further studies to advance the development of this compound.

The third study, describes an *in vitro* quiescent state of uropathogenic *E. coli* (UPEC) and bacteria-produced signals that can prevent this state. Quiescence was seen in the classic UPEC strain CFT073 only when grown on glucose M9 minimal medium agar plates seeded with $\leq 10^6$ CFU. Interestingly, this quiescent state is seen in ~80% of *E. coli* phylogenetic group B2 multilocus sequence type 73 strains, as well as 22.5% of randomly selected UPEC strains isolated from community acquired urinary tract infections in Denmark. Furthermore, it was determined that CFT073 forms a high persister cell fraction under these growth conditions. Both the persistent and quiescent states were inhibited significantly by a cocktail of lysine, tyrosine, and methionine at concentrations relevant to those in human urine. The use of CFT073 mini-Tn5 metabolic

mutants (*gnd*, *gdhA*, *pykF*, *sdhA*, and *zwf*) showed that both quiescence and persistence require a complete TCA cycle, but that the dormant states differ in that persistence requires a non-functional *rpoS* gene and quiescence does not. These results suggest that interference with these central metabolic pathways may be able to mitigate UPEC infections.

In the fifth chapter, cranberry oligosaccharides and related compounds were determined to be able to reduce the quiescent and persistent phenotypes of UPEC CFT073. This is the first report describing components of cranberry juice with the ability to modulate these important physiological aspects of UPEC and further suggests that cranberry oligosaccharides may be vital to the effectiveness of cranberry juice products in urinary tract infections.

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PREFACE

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TABLE OF CONTENTS

Abstract	ii
Acknowledgements	v
Preface	ix
Table of Contents	x
List of Figures	xii
List of Tables	xiv
 Chapter 1. Introduction	 1
References	8
 Chapter 2. Enhancement of Antibiotic Activity against Multidrug Resistant Bacteria by the Efflux Pump Inhibitor, 3,4-Dibromopyrrole-2,5-dione, Isolated from a <i>Pseudoalteromonas</i> sp.....	 14
Abstract	16
Introduction	17
Results and Discussion	21
Experimental Section	34
References	46
 Chapter 3. A Marine Bacteria-Produced Benzisoxazole with Antibacterial Activity Against Multi-Drug Resistant <i>Acinetobacter baumannii</i>	 56
Abstract	58
Introduction	59
Results	60
Discussion	64
Materials and Methods.....	66
References	76
 Chapter 4. Uropathogenic <i>Escherichia coli</i> Metabolite-Dependent Quiescence and Persistence May Explain Antibiotic Tolerance during Urinary Tract Infection	 80
Abstract	82
Introduction	83
Materials and Methods.....	85
Results	91
Discussion	105
References	112

	Legends to Figure	127
Chapter 5.	Constituents from Cranberry Juice Prevent Quiescence and Persistence in Uropathogenic <i>Escherichia coli</i>	142
	Abstract	144
	Introduction.....	145
	Results.....	147
	Discussion	150
	Methods.....	152
	References.....	156
Chapter 6.	Perspective	169
Appendix A.	Supplementary Materials for Chapter 3	172
Appendix B.	Supplemental Figures for Chapter 5	186

List of Figures

Chapter 2	
Figure 1.	Effect of 3,4-dibromopyrrole-2,5-dione on Hoechst 33342 accumulation and efflux in wild-type (AG100) and transporter-deficient (AG100A) <i>E. coli</i>53
Figure 2.	Phylogenetic and exo-metabolomic comparison of <i>Pseudoalteromonas</i> sp. in culture collection.....54
Figure 3.	Heat map displaying relative abundances of inferred compounds produced by <i>Pseudoalteromonas</i> isolates from (A) –HRESI and (B) +HRESI mode data.....55
Chapter 3	
Scheme 1.	Synthesis of 3,6-dihydroxy-benzisoxazole61
Figure 1.	Panel of compounds tested for MICs against <i>A. baumannii</i>63
Chapter 4	
Figure 1.	<i>E. coli</i> CFT073 quiescence on glucose plates.....131
Figure 2.	<i>E. coli</i> CFT073 non-quiescence on glycerol, ribose, and xylose plates ..132
Figure 3.	Prevention of quiescence by human urine and amino acids133
Figure 4.	<i>E. coli</i> CFT073 and <i>E. coli</i> MG1655 persistence134
Figure 5.	<i>E. coli</i> CFT073 persistence in the presence of amino acids135
Figure 6.	Diagram of <i>E. coli</i> central carbon metabolism136
Figure 7.	<i>E. coli</i> CFT073 <i>sdhA</i> persistence137
Figure 8.	<i>E. coli</i> CFT073 <i>gdhA</i> persistence.....138
Figure 9.	<i>E. coli</i> CFT073 <i>sdhA</i> persistence in the presence of fumarate139
Figure 10.	Rescue of <i>E. coli</i> CFT073 <i>sdhA</i> quiescence by fumarate140
Figure 11.	Quiescence of the <i>E. coli</i> CFT073 clinical isolate on glucose plates141
Chapter 5	
Figure 1.	Persister cell viability assay with Cranf1b fraction161
Figure 2.	Persister cell viability assay with 10% PGC fraction162
Figure 3.	Structure and subunit designation of unsaturated methyl galacturonic acid oligosaccharide (4-mer)163
Figure 4.	ESI-TOF MS-MS of unsaturated methyl galacturonic acid oligosaccharide (4-mer)164
Figure 5.	Inhibition of quiescence using cranberry oligosaccharides165
Figure 6.	Amide-80 HPLC chromatogram of 10% PGC166
Figure 7.	C18 Reverse-phase HPLC chromatogram of iridoid glycosides purification.....167

Figure 8.	LC/MS analysis of 10% PGC fraction.....	168
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Appendix A

Figure S1.	Chemical structures of compounds 11 and 12	181
Figure S2.	LC/MS comparison of isolated 1 to synthetic 1	182
Figure S3.	¹ H NMR of isolated 1	183
Figure S4.	¹ H NMR of synthesized 1	184
Figure S5.	¹³ C NMR of synthesized 1	185

Appendix B

Figure S1.	High resolution ESI-TOF-MS of 4	187
Figure S2.	Structures of Iridoid glycosides 1-3	188
Figure S3.	¹ H NMR spectrum of 10% PGC	190
Figure S4.	HSQC spectrum of 10% PGC.....	191
Figure S5.	¹ H NMR spectrum of 4	192
Figure S6.	¹³ C NMR spectrum of 4	193
Figure S7.	COSY spectrum of 4	194
Figure S8.	TOCSY spectrum of 4	195
Figure S9.	HSQC spectrum of 4	196
Figure S10.	HMBC spectrum of 4	197
Figure S11.	NOESY spectrum of 4	198
Figure S12.	¹ H NMR spectrum of 1	199
Figure S13.	¹³ C NMR spectrum of 1	200
Figure S14.	COSY spectrum of 1	201
Figure S15.	TOCSY spectrum of 1	202
Figure S16.	HSQC spectrum of 1	203
Figure S17.	HMBC spectrum of 1	204
Figure S18.	¹ H spectrum of 2	205
Figure S19.	COSY spectrum of 2	206
Figure S20.	TOCSY spectrum of 2	207
Figure S21.	HSQC spectrum of 2	208
Figure S22.	¹ H spectrum of 3	209
Figure S23.	COSY spectrum of 3	210
Figure S24.	TOCSY spectrum of 3	211
Figure S25.	HSQC spectrum of 3	212
Figure S26.	HR-MS of 1	213
Figure S27.	HR-MS of 2	214
Figure S28.	HR-MS of 3	215

List of Tables

Chapter 2

Table 1.	Minimum inhibitory concentration potentiation of 3,4-dibromopyrrole-2,5-dione on various classes of antibiotics against <i>E. coli</i> strains overexpressing efflux pumps	24
Table 2.	Hoechst 33342 accumulation and efflux in the presence of 3,4-dibromopyrrole-2,5-dione	26

Chapter 3

Table 1.	¹ H and ¹³ C-NMR data comparison of 3,6-dihydroxy-1,2-benzisoxazole to synthetic and published data	61
Table 2.	Minimum inhibitory concentration values of tested compounds against included panel of <i>A. baumannii</i>	63

Chapter 4

Table 1.	Bacterial strains	121
Table 2.	PCR primer sequences for amplifying mutant genes containing mini-Tn5 Km insertions	124
Table 3.	Free amino acid composition of 50-fold concentrated <i>E. coli</i> MG1655 and <i>E. coli</i> CFT073 supernatants and human urine	125
Table 4.	<i>E. coli</i> CFT073 persister cells relative to persister cells for other <i>E. coli</i> strains	126

Appendix A

Table S1.	¹ H and ¹³ C NMR data of 12 and comparison to published data	179
Table S2.	<i>A. baumannii</i> strain characteristics and resistance profiles	180

Appendix B

Table S1.	¹³ C and ¹ H NMR chemical shifts of 4	186
Table S2.	¹³ C and ¹ H NMR chemical shifts of 1 , 2 and 3	189

CHAPTER 1

Introduction

Bacterial infections and the post-antibiotic era

Infectious disease remains a significant threat to human health in the United States and worldwide. In 2010, 117,716 deaths were attributed to infections of bacterial, viral, or parasitic origins in the US.¹ As a category, this ranks infectious disease as the fourth most deadly form of illness behind only cardiovascular disease, cancer, and respiratory disease, and ahead of diabetes and Alzheimer's disease. Worldwide, infectious disease has an even larger impact, accounting for 10 million deaths in 2011.² This ranks it as the second most lethal disease category behind only cardiovascular disease.² A growing concern in the treatment of infectious disease is the drastic increase in antimicrobial resistance. This is present in bacteria, viruses, and fungi, and is complicating the treatment of infectious disease considerably. In fact, certain pathogenic bacteria now exist that are resistant to nearly all treatment options, forecasting the so-called 'post-antibiotic era.'³⁻⁶ The problem of antimicrobial resistance is immense with an estimated 700,000 deaths worldwide each year, and these numbers are increasing.⁴ It is estimated that in 2050 there will be 10 million deaths due to antimicrobial resistance globally in a single year.^{4, 7} These deaths would lead to an estimated net loss of 100 trillion dollars of economic output.^{4, 7} Deaths due to infections are not the only clinical consequence of a post-antibiotic era. Surgery, chemotherapy, and organ transplantation rely heavily on antibiotics to facilitate their success.^{4, 7} Continuing on the trend of increasing antimicrobial resistance would lead to a catastrophic change in lifestyle both economically and medically.

Among the most threatening problems in antimicrobial resistance are bacterial infections involving multi-drug-resistant (MDR) pathogens. These are especially a problem in the United States, where 23,000 people die each year due to MDR bacterial infections.³ This is an especially common problem in the hospital setting where antimicrobial resistance is difficult to eradicate and is seen in common nosocomial pathogens.⁸ These pathogens that have been identified as high priorities for research are referred to as ‘ESKAPE’ pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species).⁹ These organisms are in general difficult to treat and can cause significant morbidity and mortality.^{9, 10} With the exclusion of *E. faecium* and *S. aureus*, the ESKAPE pathogens are Gram-negative bacteria. This is important because there are in general fewer treatment options for Gram-negative bacteria and developing antibiotics against these bacteria has been historically less successful.¹¹ The World Health Organization recently released a ranking of highest priority bacteria for antibiotic development, and all three critically important organisms are MDR Gram negative bacteria.¹²

A major reason that the problem of antimicrobial resistance is increasing is the dearth of new antibiotics being approved or researched. Since 1998, only four systemic antibiotics (linezolid, daptomycin, tigecycline, and telavancin) have been approved that have novel mechanisms of action.¹⁰ To combat this issue, antibiotic stewardship programs can help, but long term solutions exist in the drug development arena. In 2010, the Infectious Disease Society of America (IDSA) launched a campaign known as the “10 x ’20 Initiative” that called for the development 10 new antibiotics approved by

2020.¹⁰ Currently we are falling well short of the goal, with only four new antibiotics being approved since the initiative and none of these with activity against the important Gram-negative bacteria.¹³ Additionally, the current pipeline of antibiotics contains only five drugs in phase II or III development with target indications of MDR Gram negative infections.¹³ There is a clear void in the future treatment of infectious disease especially for MDR bacterial infections, and without changes to the current course, a post-antibiotic era is likely.

Bacterial mechanisms of resistance

Bacteria have developed mechanisms to avoid the toxic effects of antibiotics, causing resistance. Inhibiting or circumventing these biological functions can restore the activity of the antibacterial drug. Understanding the mechanisms of resistance is crucial to developing agents that can restore the potency of antibiotics, especially in ESKAPE pathogens and Gram-negative bacteria. Resistance mechanisms fall into these general categories: drug alteration/inactivation, reduced drug intracellular accumulation, binding site modification, and biofilm formation.⁸

Drug altering or inactivating enzymes are well studied and therapies have been developed that can inhibit these enzymes. A well-studied example is the β -lactamase type enzymes which are capable of inactivating β -lactam antibiotics such as penicillins, cephalosporins, monobactams, and carbapenems.¹⁴ These enzymes have been the targets of β -lactamase inhibitor drugs such as clavulanic acid, tazobactam, and sulbactam, all of which are used in combination with β -lactam antibiotics to successfully treat bacteria that produce β -lactamases.^{15, 16} While these approaches have been successful, the emergence

of extended spectrum β -lactamases (ESBLs) such as New Delhi β -lactamase (NDM-1) and oxacillin hydrolyzing enzymes (OXA) that are resistant to β -lactamase inhibitors and confer resistance to most β -lactams is concerning.^{15, 17, 18}

When bacteria are able to reduce the intracellular concentration of antibiotics, this is frequently due to reduced production of channels that transport antibiotics into the cell, or the up-regulation of efflux pumps able to eject antibiotics.⁸ Efflux pumps expel antibiotics from the bacterial cytoplasm before they can reach their site of action.^{19, 20} These pumps can expel a broad range of substrates by coupling the efflux with a proton or other ion.²¹ Certain efflux pumps, such as the resistance nodulation cell division (RND) family pump AcrAB, have been suggested as biomarkers for the MDR phenotype since they confer resistance to ≥ 5 antibiotics.²² Efflux pumps also contribute to the resistance present in the ESKAPE pathogens.^{23, 24} The ability to inhibit efflux pumps with a drug adjuvant to antibacterial therapy could help overcome resistance and treatment failures. There has been progress in identifying efflux pump inhibitors (EPIs) of Gram-positive pathogens such as *S. aureus*, but there is a sizable knowledge gap for Gram-negative efflux pumps.²⁴ Discovery of EPIs for Gram-negative bacteria is greatly needed to help supplement the already bare pipeline of new therapies for these problematic pathogens.

Persistence and quiescence of bacterial pathogens

The phenomenon of bacterial persistence is a rapidly expanding research area in the field of antibiotic development. Similar to resistance, bacterial persistence makes the treatment of infections much more difficult. Persistence is characterized by dormant

bacterial cells (persister cells) that are highly tolerant to antibiotics.²⁵ When these cells are isolated, re-cultured, and tested for antibiotic susceptibility, they show full susceptibility, indicating these are not resistant mutants. In contrast, antibiotic resistant bacteria will show a decrease in antibiotic susceptibility (require higher concentrations of antibiotics to inhibit the growth of the bacteria). Persister cells are implicated as the causative phenotype in chronic infections such as recurrent urinary tract infections (UTIs), infections in cystic fibrosis, and tuberculosis.²⁶⁻²⁸ In addition to chronic infections, persister cells are commonly associated with biofilms, which can allow them to evade the immune system as well as exhibit antibiotic tolerance, causing antibiotic treatment failures.^{25, 26, 28}

Uropathogenic *E. coli* (UPEC) is an ideal organism to study persistence. 85% of all UTIs are caused by UPEC and reinfection is common.²⁹ Studies have shown that UPEC are able to invade bladder and kidney epithelial cells during a UTI.^{30, 31} Following invasion, the UPEC can either establish an intracellular bacterial community that evades host immune defenses (in acute infection) or a quiescent intracellular reservoir (for recurrent infections).³¹ The quiescent intracellular reservoir can remain viable for months and cause reinfection following exfoliation of UPEC containing epithelial cells. Drugs capable of inhibiting persistence and thereby inhibiting reinfection would be valuable tools in the treatment of UTIs caused by UPEC. Additionally, compounds that reduce persistence in UPEC could help identify targets and mechanisms necessary for persister cell formation in other important bacterial pathogens. This could lead to new drugs and improved treatment outcomes in challenging bacterial infections.

Natural products as drug leads and antibiotics

Natural products represent both a classical source of drug molecules and a promising future resource of novel medicines.^{32, 33} From the years of 1981-2014, 1211 small molecule drugs were approved for use, and of those 791 (64%) were either natural products or inspired by natural products.³² More importantly, 59% of all drugs approved to treat bacterial infections were naturally derived or inspired.³² The fraction increases to 74% when you exclude vaccines and look at only small molecule drugs (e.g., antibiotics), indicating that natural products are by far the most significant source of antibiotics available. Of the 12 major classes of antibiotics, nine of these, including β -lactams, macrolides, tetracyclines, aminoglycosides, glycopeptides, and lipopeptides, contain a natural product derived structure.³⁴

Microorganisms have also been prolific producers of antibacterial compounds. It is estimated that 16,500 antibiotic secondary metabolites have been discovered from microorganisms and roughly 12,000 of those have antibacterial effects.³⁵ While this seems like a vast amount of compounds, genome sequencing has revealed that many of the biosynthetic gene clusters are dormant under laboratory conditions, potentially indicating that we have only scratched the surface of antibiotic compounds from microorganisms.^{35, 36} An additional resource is the many ‘unculturable’ species. It is estimated that upwards of 90% of all terrestrial microorganisms, and >99% of all aquatic microorganisms do not grow under normal laboratory conditions.^{35, 37} The amount of species diversity yet unstudied is vast, and we are now developing techniques to culture these ‘unculturable’ microorganisms.^{37, 38} It is clear that the most prolific producers of antibiotics, microorganisms, are still largely unstudied and could yield thousands more

antibiotics as yet undiscovered. With the current shortcomings in the development of new anti-infective agents, additional investigations of natural products, especially those from microorganisms should be pursued.

Marine microorganisms as sources of bioactive natural products

There is significant data to suggest the promise of marine organisms as potential sources of bioactive compounds, including antibiotics. In a marine environment, an average of 10^6 bacterial cells and 10^3 fungal cells exist per milliliter of seawater which creates a constant and mobile threat of infection to macroorganisms, as well as competition between microorganisms for survival.³⁴ Investigations of marine organisms have uncovered compounds in diverse classes of secondary metabolites (ribosomal peptides, non-ribosomal peptides, polyketides, alkaloids, and terpenes) that possess potent bioactivities.³⁴ Marine sediments also harbor vast microbial diversity (10^8 cells in a gram of wet sediment) that has been largely unstudied.^{34, 39} Compared to terrestrial studies, the search for antibiotics from the marine environment is still in its infancy.³⁴ The marine environment comprises a vital resource of biodiversity that should be further explored for new antibiotics.

Natural products discovered from the marine environment span a large variety of organisms, but microorganisms show significant potential. Because terrestrial microorganisms have been such a significant source of antibacterial compounds, it stands to reason that marine microorganisms would also be prolific producers of bioactive molecules. 491 (42%) of the new compounds isolated from the marine environment in 2013 were isolated from microorganisms indicating they are already a major source of

marine chemistry.⁴⁰ These compounds exhibit diverse bioactivities including antibacterial, anticancer/cytotoxic, anti-quorum sensing, antiviral, anti-inflammatory, immunosuppressant, antiprotozoal, and antifungal.⁴⁰⁻⁴² Some antibacterials discovered from marine microorganisms include non-ribosomal peptides (bogorol A, emericellamide A, and thiocoraline), polyketides (abyssomycin C and pestalone), and alkaloids (marinopyrrole A).³⁴ Additionally, while the macroorganisms such as sponges, algae, and tunicates represent a significant source of chemistry, it is proposed that microorganisms acting in symbiotic relationships are responsible for at least a portion of the identified novel chemistry.^{43, 44} There are currently 20 drugs (molecules in clinical trials or approved by the FDA) either isolated from a marine source or inspired by marine chemistry.⁴⁵ It has been proposed that 17 are produced by microorganisms associated with the macroorganisms from which those drugs were originally isolated.⁴⁵ Thus, marine microorganisms show significant potential as a resource for new antibacterial and other bioactivities that might be useful in the future treatment of drug resistant bacterial infections.

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CHAPTER 2

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Enhancement of Antibiotic Activity Against
Multidrug Resistant Bacteria by the Efflux
Pump Inhibitor, 3,4-Dibromopyrrole-2,5-dione,
Isolated from a *Pseudoalteromonas* sp.

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Dedicated to Dr. William Fenical of Scripps Institution of Oceanography, University of
California-San Diego, for his pioneering work on bioactive natural products

ABSTRACT

Members of the Resistance Nodulation Cell Division (RND) of efflux pumps play essential roles in multidrug resistance (MDR) in Gram-negative bacteria. Herein, we describe the search for new small molecules from marine microbial extracts to block efflux and thus restore antibiotic susceptibility in MDR bacterial strains. We report the isolation of 3,4-dibromopyrrole-2,5-dione, an inhibitor of RND transporters from Enterobacteriaceae and *Pseudomonas aeruginosa*, from the marine bacterium, *Pseudoalteromonas piscicida*. 3,4-Dibromopyrrole-2,5-dione decreased the MICs of two fluoroquinolones, an aminoglycoside, a macrolide, a beta-lactam, tetracycline, and chloramphenicol between 2 and 16-fold in strains overexpressing three archetype RND transporters (AcrAB-TolC, MexAB-OprM, and MexXY-OprM). 3,4-Dibromopyrrole-2,5-dione also increased the intracellular accumulation of Hoechst 33342, in wild-type, but not in transporter deficient strains; and prevented H33342 efflux ($IC_{50} = 0.79 \mu\text{g/mL}$ or $3 \mu\text{M}$), a hallmark of efflux pump inhibitor (EPI) functionality. A metabolomic survey of 36 *Pseudoalteromonas* isolates mapped the presence of primarily brominated metabolites only within the *P. piscicida* phylogenetic clade where a majority of antibiotic activity was also observed, suggesting a link between halogenation and enhanced secondary metabolite biosynthetic potential. In sum, 3,4-dibromopyrrole-2,5-dione is a potent EPI and deserves further attention as an adjuvant to enhance the effectiveness of existing antibiotics.

The emergence of multidrug resistant (MDR) Gram-negative pathogens, including Enterobacteriaceae and *Pseudomonas aeruginosa*, has become a substantial threat to treating infectious disease. Twenty percent of all deaths globally are the result of bacterial infections,¹ and hospital-acquired infections are the sixth leading cause of mortality in the U.S.,² with 23,000 deaths per year due to MDR infections in the U.S. alone.³ The rapid decline in antibiotic effectiveness has led some clinicians to estimate the future utility of available antibiotics to be limited to a few years, in some cases, against MDR strains.⁴ Developing tools to battle these emerging MDR Gram-negative pathogens deserves priority status,⁵ as they now account for nearly 70% of infections in intensive care units in the U.S.² Alarming, many Gram-negative clinical strains are becoming resistant to nearly all antibiotics including aminoglycosides, cephalosporins, fluoroquinolones and carbapenems,³ leaving few antibiotics in the therapeutic repertoire to treat these infections.⁶⁻⁸ In the search for new antimicrobials, random screening of libraries of synthetic or natural products are estimated to have a primary hit-rate of up to 1,000-fold lower against Gram-negative than for Gram-positive bacteria.⁹ Of the antibiotics approved by the FDA from 1998 to 2005, including those in clinical trials, and various stages of preclinical development, most lack appropriate activity against any Gram-negative bacteria.^{6, 7} Making incremental improvements to the chemical scaffolds of existing antibiotics is at best a short-term strategy for the impelling need for both new drugs and novel approaches to combat multidrug resistant pathogens.¹⁰ Consequently, developing compounds targeting the resistance mechanisms themselves is warranted (e.g. the clinically proven beta-lactam/beta-lactamase inhibitor cocktail, amoxicillin/clavulanic

acid),⁴ thereby i) obviating the emergence of resistance, and ii) regaining antibiotic potency.

The rapid spread of resistance is, in part, a result of constitutive over-expression of transmembrane efflux pumps that expel antibiotics before they can reach their intracellular target.¹¹ Members of the Resistance Nodulation Cell Division (RND) superfamily of MDR pumps have been implicated in the high intrinsic resistance of Gram-negative species,^{12, 13} whose tripartite RND pumps recognize and expel a broad range of substrates (including antibiotics, charged and neutral molecules, organic solvents, lipids, bile salts, and quorum signal molecules),⁹ via a coupled exchange with protons or ions.¹⁴ Permanent overexpression of RND pumps leads to multidrug resistance in bacteria,¹⁵ while their deletion restores antibiotic susceptibility¹⁶ further confirming this transporter is an important therapeutic target. Polyspecificity of RND pumps is central to the emergence and spreading of efflux-mediated resistance, as these pumps subsequently allow for acquisition of additional resistance mechanisms,^{9, 17} and have a significant role in bacterial pathogenicity/virulence, invasion, adherence and host colonization.¹⁴ In drug resistant *Escherichia coli*, *Salmonella enterica*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae*, overproduction of AcrAB-TolC pump and its homologue MexAB-OprM/MexXY-OprM in *Pseudomonas aeruginosa* are the main RND archetypes reported in clinical isolates.^{11, 17, 18} RND transporters are found in both prokaryotes and eukaryotes; however, homology between bacterial and human RND proteins is negligibly low (16% identity), suggesting minimal overlap in RND transporter substrate specificities.⁷ In addition, clinical isolates with more resistant MDR phenotypes (resistant to ≥ 5 antibiotics, including 100% fluoroquinolone resistance), are more likely

to overexpress the RND pump, AcrAB, suggesting this RND pump maybe a biomarker of MDR,¹⁹ making these efflux pumps “key” targets for the development of an efflux pump inhibitor (EPI) as an adjuvant to existing antibiotics.²⁰

Our search for small molecule EPIs from the microbial realm has been aided by the fact that natural products have often been selected precisely for their ability to penetrate both outer and inner membranes of Gram-negative bacteria.²¹ A countermeasure by antibiotic-producing microbes is to co-evolve inhibitors of their competitor’s resistance mechanisms to enhance the efficacy of their own antibiotics,²¹ exemplified by the *Streptomyces* spp. producing both beta-lactam antibiotics and the beta-lactamase inhibitor clavulanic acid.²² There is substantial evidence that marine bacteria produce cocktails of both antibiotics to control surface colonization,^{23,24} and nontoxic secondary metabolites capable of quenching quorum sensing-controlled activities in other species.²⁵ However, to our knowledge no systematic study has screened for EPIs from marine microbial exudates (i.e., compounds excreted into the extracellular medium) against RND pumps. Regardless, our approach is validated by previous screening of terrestrially-derived microbial fermentations which resulted in two new natural product EPIs targeting MexAB-OprM from *Streptomyces* (EA-371 α and EA-371 δ) potentiating levofloxacin minimum inhibitory concentrations (MICs) 4-fold and 8-fold, respectively.²⁶ The microbial EPI, MP-601,205 is currently used to treat *P. aeruginosa* respiratory infections in cystic fibrosis patients.¹⁴ Thus far, a diverse set of natural product chemical scaffolds (including polyphenols, flavones, flavonols, flavonolignans, flavonoids, diterpenes, triterpenoids, oligosaccharide-glycosides, and pyridines) have been validated as EPIs in

Gram-positive bacteria such as *Staphylococcus aureus*.²⁷ A remaining challenge is the discovery of EPIs targeted toward Gram-negative efflux pumps.²⁷

We hypothesized that microorganisms obtained from the marine environment produce EPIs as regulators of diverse ecological interactions, and as such present a unique bio-prospecting opportunity. In the present paper our objectives were: (i) to screen our in-house chemical library to identify marine microbial isolates capable of reducing antibiotic MICs ≥ 4 -fold in three strains overexpressing three archetype RND transporters (AcrAB-TolC, MexAB-OprM, and MexXY-OprM) common in Gram-negative pathogens, (ii) to isolate and chemically characterize the putative EPI lead, (iii) to evaluate the potential of our lead molecule to potentiate the activity of various classes of antibiotics, (iv) to quantify the inhibitory activity of this molecule in functional whole cell accumulation and efflux assays, and (v) to compare the exo-metabolomic fingerprints (i.e., the relative abundances of chemical features with unique m/z values and retention times) of EPI-producing *Pseudoalteromonas* strains versus other related members of the genus, thereby linking metabolite diversity with genetic relatedness. We report herein the isolation, identification, and demonstration of EPI functionality of 3,4-dibromopyrrole-2,5-dione, an inhibitor of RND transporters from Enterobacteriaceae and *P. aeruginosa*, having been isolated from the marine bacterium, *Pseudoalteromonas piscicida*. Our paper also demonstrates that the production of halogenated secondary metabolites is associated with those *Pseudoalteromonas* clades harboring antibiotic compounds and EPIs; further highlighting that marine microbial sources, especially marine Gram-negative bacteria, represent a tractable source of new chemical scaffolds for EPI development.

RESULTS AND DISCUSSION

Isolation and Identification of 3,4-Dibromopyrrole-2,5-dione. Crude exudate extracts from a total of 1308 marine microbial isolates were screened in the *p*-Iodonitrotetrazolium chloride (INT) assay to assess MDR reversal potential. This initial screening identified 36 marine microbial isolates of diverse phylogenetic affiliation (2.8% hit rate) capable of potentiating (i.e., reducing the antibiotic MIC by at least 4-fold) the activity of chloramphenicol and/or erythromycin when tested against *E. coli* MDR strains AG102, MG1655 Δ BC/pABM, and MG1655 Δ BC/pXYM. Phylogenetic analysis of 16S rRNA sequences indicated seven isolates of these 36 were *Pseudoalteromonas* sp. (A197, A198, A256, A746, A754, A757, B126) (Figure S1, Table S1, Supporting Information). Isolate B126 could not be revived from frozen culture; therefore it was dropped from further analysis. Crude extracts displaying an initial positive hit in one or more test *E. coli* strains in the bacterial susceptibility assay were prioritized for further testing. The INT assay was performed in the presence and absence of antibiotic to establish the MIC of the crude extract (tested in a serial dilution series) and determine if EPI-like activity was present by examining the minimal effective concentration of the crude extract required to potentiate the antibiotic MIC at least 4-fold. These experiments indicated isolates A197, A198, and A256 produced antibiotics only, while A746, A754, and A757 demonstrated EPI potential and antibiotic activity (data not shown). The strongest EPI-like activity was seen for A757, therefore this isolate was chosen for further study. Bioassay-guided fractionation of the crude extract generated from 16.5 L of A757 exudates resulted in the isolation of 3,4-dibromopyrrole-2,5-dione. The structure of 3,4-dibromopyrrole-2,5-dione was established on the basis of NMR spectroscopic and

LC-MS data in comparison with an authentic standard (Figure S2-6, Supporting Information). To our knowledge, this paper represents the first description of 3,4-dibromopyrrole-2,5-dione from a microbial source, although the original report of its isolation from nature was from the marine sponge *Axinella brevistyla* collected in western Japan, where it was reported to exert modest antifungal activity and cytotoxicity against murine lymphocytic leukemia cells.²⁸ However, since over 50% of a sponge's biomass can be attributed to microorganisms, it is conceivable that the origin of 3,4-dibromopyrrole-2,5-dione may be from sponge-associated microorganisms.²⁹

Antibiotic Potentiation Activity by 3,4-Dibromopyrrole-2,5-dione. The checkerboard assay was used to determine whether 3,4-dibromopyrrole-2,5-dione potentiates the activity of two fluoroquinolones (ciprofloxacin; CIP, and levofloxacin; LEV), an aminoglycoside (kanamycin; KAN), a macrolide (erythromycin; ERY), two β -lactams (oxacillin; OXA, and piperacillin; PIP), tetracycline (TET) and chloramphenicol (CHL), known substrates for RND transporters,^{17, 30-32} against our test strains of MDR *E. coli*. The checkerboard method is so named due to the pattern of wells formed by dilutions of the antibiotic and test compound being examined, at concentrations above, at, and below their MICs. Interactions between 3,4-dibromopyrrole-2,5-dione and various antibiotics were classified based upon the fractional inhibitory concentration index (FICI), where FIC of < 0.5 is synergistic, and indicative of the presence of an EPI; $0.5-1$ is indifferent; and >1 is antagonistic. The results, shown in Table 1, demonstrate 3,4-dibromopyrrole-2,5-dione decreases the MICs of various antibiotic classes between 2 and 16-fold, with corresponding fractional inhibitory concentration indices (FICI) indicating synergistic activity of 3,4-dibromopyrrole-2,5-dione with all antibiotics tested with the exception of

piperacillin. 3,4-Dibromopyrrole-2,5-dione exhibited antibacterial activity at concentrations $>100\text{ }\mu\text{g/mL}$ for all three *E. coli* strains overexpressing efflux pumps. The greatest decrease in antibiotic MICs was seen for erythromycin, where 3,4-dibromopyrrole-2,5-dione decreased the antibiotic MICs between 8 and 16-fold.

Table 1. 3,4-dibromopyrrole-2,5-dione potentiates the antibacterial activity of various classes of antibiotics against *E.coli* strains that over-express efflux pumps.

Strain	DBPD		CHL		CIP		ERY		KAN		LEV		OXA		PIP		TET	
	MIC ^a	FICI ^f	MIC ^a	FICI ^f	MIC ^a	FICI ^f	MIC ^a	FICI ^f	MIC ^a	FICI ^f	MIC ^a	FICI ^f	MIC ^a	FICI ^f	MIC ^a	FICI ^f	MIC ^a	FICI ^f
AG102	256	24	6 (64)	0.313	0.13	0.063(2)	0.500	128	16 (128)	0.375	16	4 (2)	0.266	0.13	0.03 (128)	0.356	>650	650 (32)
ΔBC/pABM	256	24	6 (128)	0.313	0.03	0.015 (64)	0.750	32	2 (64)	0.188	2	0.5 (128)	0.750	0.13	0.06 (64)	0.750	>1300	n/a
ΔBC/pXYM	128	0.75	0.09 (64)	0.188	0.016	0.002 (32)	0.188	32	2 (32)	0.313	0.25	0.13 (32)	0.750	0.13	0.02 (32)	0.188	>1300	650 (64)
MIC ^a , MIC of antibiotic alone; MIC ^b , MIC of antibiotic in the presence of DBPD (concentration of DBPD (μg/mL) in parentheses); FICI ^f , Fractional inhibitory concentration index																		
DBPD, 3,4-dibromopyrrole-2,5-dione																		

DBPD, 3,4-dibromopyrrole-2,5-dione

Hoechst 33342 Accumulation and Efflux Inhibition. To verify that inhibition of efflux, and not membrane permeabilization, is the mechanism by which 3,4-dibromopyrrole-2,5-dione potentiates antibacterial activity, we determined whether 3,4-dibromopyrrole-2,5-dione was capable of causing the accumulation of the fluorescent efflux pump substrate (Hoechst 33342) in the wild-type, *E. coli* AG100 expressing the AcrAB-TolC pump; and conversely confirm the *E. coli* mutant AG100A lacking the pump would not be affected. Upon entering the bacterial cell, H33342 becomes fluorescent once bound to the DNA minor groove. As shown in Figure 1A, addition of 3,4-dibromopyrrole-2,5-dione at concentrations ranging from 0.06 – 64 $\mu\text{g/mL}$, resulted in a dose-dependent increase in intracellular H33342 fluorescence, consistent with an EPI effect. Concentrations of 3,4-dibromopyrrole-2,5-dione $\geq 1 \mu\text{g/mL}$ were equal to or more effective than the reference inhibitor PA β N (15.6 $\mu\text{g/mL}$) in causing H33342 accumulation. We reasoned that antibiotic-sensitive mutant *E. coli* (AG100A), lacking the target RND pump of 3,4-dibromopyrrole-2,5-dione would not be affected by the compound. Results displayed in Figure 1B, show there is no effect of 3,4-dibromopyrrole-2,5-dione on H33342 fluorescence in AG100A, which is consistent with the hypothesis that RND pumps are the target of 3,4-dibromopyrrole-2,5-dione and indicates MDR reversal is limited to efflux pump expressing *E. coli* strains. In addition, if the mechanism of action of 3,4-dibromopyrrole-2,5-dione was via membrane permeabilization, we would have seen a dose-dependent increase in H33342 accumulation regardless of test bacterial strain, which we did not observe.

Demonstration of efflux activity in AG100 requires that accumulation of H33342 has taken place. Following H33342 “loading”, monitoring of efflux was initiated in the

presence of increasing concentrations of 3,4-dibromopyrrole-2,5-dione (0.06 – 64 $\mu\text{g/mL}$) in medium containing glucose at 37°C. The near complete inhibition of H33342 efflux was observed in comparison to control wells (Figure 1C) indicating efflux competent bacterial cells were not capable of transporting H33342 in the presence of 3,4-dibromopyrrole-2,5-dione. Concentrations of 3,4-dibromopyrrole-2,5-dione as dilute as 0.06 $\mu\text{g/mL}$ showed significant ($p < 0.05$) H33342 efflux inhibition in comparison to solvent controls (Table 2). The IC_{50} value for 3,4-dibromopyrrole-2,5-dione in the efflux assay was calculated to be 0.79 $\mu\text{g/mL}$ or 3 μM . A comparison of bacterial RND pump EPIs reveals that compounds, including 3,4-dibromopyrrole-2,5-dione, contain hydrophobic ring systems that presumably interact with hydrophobic residues located near or in the substrate binding site.³³

Table 2. Hoechst 33342 accumulation and efflux of in the presence of 3,4-dibromopyrrole-2,5-dione

Compound ($\mu\text{g/mL}$)	Accumulation				Efflux	
	AG100 ^a		AG100A ^b		AG100 ^a	
	Mean (RFU)	S.D.	Mean (RFU)	S.D.	Mean (RFU)	S.D.
DBDP						
64	1208***	20	1090	10	-68***	54
16	1165***	8	1103	14	-34***	58
4	1130***	11	1175	8	-101***	34
1	1006***	4	1065	5	-247***	30
0.25	796***	12	1003	17	-351***	7
0.063	758	9	1016	15	-422*	37
Hoechst only	746	16	1038	10	-472	13

^aAG100, wild-type *E. coli* K-12 strain

^bAG100A, ΔacrAB mutant

Final mean values from last four minutes of the assay, compared using a one-way ANOVA with Dunnett's multiple comparison test comparing treatment versus Hoechst H33342 control. The level of significance is indicated by asterisks (*** $p < 0.0001$, * $p < 0.05$, $n=8$) IC_{50} value calculated from efflux data was 0.79 $\mu\text{g/mL}$ or 3 μM (95% confidence interval 0.69-0.91 $\mu\text{g/mL}$).

3,4-Dibromopyrrole-2,5-dione also is known to react with disulfide linkages in the presence of a strong reducing agent such as (tris(2-carboxyethyl)phosphine).³⁴ It is conceivable, but unlikely, that 3,4-dibromopyrrole-2,5-dione is displacing and coordinating certain accessible, reduced disulfide linkages in the RND pumps and permanently modifying their conformation by this crosslinking type displacement; however, the lack of antibiotic activity and range of pharmacokinetics argue against this type of action. Although the subject of another study, experiments employing halogenated and non-halogenated maleimides will be informative in terms of mode of action.

Chemophylogenetic Analysis of Extracts from *Pseudoalteromonas* Isolates.

Analysis of our untargeted -HRESI metabolomics comparison indicated the presence of many halogenated features, the distributions of which differed greatly across *Pseudoalteromonas* clades (Figure 2B and 2C, Figure S7, Table S2, Supporting Information). Principle component analysis (PCA) of -HRESI mode data (n = 1112 chemical features present in *Pseudoalteromonas* samples at concentrations 10x those in the media only blank) revealed that most *Pseudoalteromonas* clades were chemically similar overall with the exception of two clades, IV and VI, which were distinguishable from the other clades based on the first two principal components of a four component model (capturing > 60% of the total variance) (Figure 2B). Of the two clades distinguishable via PCA, Clade IV contained the EPI producing strain A757 and two additional strains with EPI activity based on dilution series testing (A746 and A754), while Clade VI contained two strains (A197 and A198) with antibacterial activity only (Table S1, Supporting Information). In summary, the PCA analysis of -HRESI data

revealed those strains with the most disparate chemistry (designated by Clades IV and VI), corresponded to the majority of strains with antibiotic activity against MDR *E. coli*.

In order to discern which chemical features were unique to Clade IV (and subsequently strain A757), we examined chemical feature loadings on the first principal component. It is important to note that great care must be taken to avoid the assumption that any of these features alone would be significantly differentially produced by *Pseudoalteromonas* strains,³⁵ however, examining the loadings is still useful and will likely reveal suites of compounds with varying concentrations in different *Pseudoalteromonas* extracts. Of the 1112 features, 221 chemical features had small negative loadings (<-0.02) within the first principal component and highly positive loadings (>0.02) on the second principal component (Figure S7, Supporting Information), and were therefore likely candidates to distinguish Clade IV from the remaining clades (Figure 2B). Further analysis of these 221 features indicated that i) several shared retention times, and ii) had masses ~ 2 amu apart, suggesting that many chemical features were isotopes of halogenated metabolites. We determined that of these 221 features, 129 were isotopes of 46 individual brominated metabolites based upon both isotopic distribution and shared retention time (Table S2, Figures S8-30, Supporting Information).

Of the 46 halogenated metabolites found, all were exclusively produced by members of Clade IV (Figure 2C), demonstrating that there is a distinct *Pseudoalteromonas* chemotype characterized by halogenation of the exo-metabolome. Moreover, this “halogenome” appears to be dominated by brominated metabolites, with only some metabolites (<10) that are additionally chlorinated (Table S2, Supporting Information). In addition, we observed several other brominated compounds ($\sim 30-40$) based upon isotopic

signatures produced by A757 that were not accounted for in the initial 221 chemical features (screened based on their loadings), suggesting that a large percentage of A757 exo-metabolome may be subject to halogenation (Tables S2 and S3, Supporting Information). Relative concentrations of halogenated compounds from Clade IV appeared different even within members of the same species (Figure 2C), indicative of intra-species chemical diversity and further sub-clustering among isolates of the same species (A757, A754, A746 and B149) a phenomenon previously described for *P. luteoviolacea* strains.³⁶ Taken together, these results indicate that the production of halogenated compounds could be a biomarker for marine isolates with enhanced biosynthetic potential. Of the four strains in our culture collection designated as belonging to Clade IV, A757 and A754 produced all 46 metabolites, whereas strains A746 and B149 produced only select halogenated metabolites, including 2,3,4,5-tetrabromopyrrole, a known weak antibiotic from *Pseudoalteromonas* spp.,³⁷ (Figure 2C). 2,3,4,5-Tetrabromopyrrole appears to be the most abundant brominated compound we observed (retention time 20.8 min, major ion m/z 381.6722, Figure 2C, Table S2, Supporting Information) and present in all members of Clade IV. Because of the dominance of 2,3,4,5-tetrabromopyrrole in our samples and its shared carbon skeleton with 3,4-dibromopyrrole-2,5-dione, we tested an authentic standard of 2,3,4,5-tetrabromopyrrole in EPI functionality assays and determined 2,3,4,5-tetrabromopyrrole is not responsible for the EPI activity of strain A757 (data not shown). We also determined the presence of 2,3,4-tribromopyrrole (retention time 20.1 min, major ion m/z 301.7639, Figure 2C, Table S2, Supporting Information) present in A757, A754 and A746, but not in B149). 2,3,4-

Tribromopyrrole is reported to be produced by *P. luteoviolacea*,³⁸ found within Clade IV, and is a known feeding deterrent in marine systems.³⁹

The limit of detection of 3,4-dibromopyrrole-2,5-dione with an authentic standard was established to be 11.1 ng/mL. Our yield from 16.5L of A757 culture was on the order of 1 mg/L of 3,4-dibromopyrrole-2,5-dione. 3,4-Dibromopyrrole-2,5-dione should have been detected in our crude that was initially screened for antibiotic activity, however, we did not see this ion until culture scale-up and further purification, suggesting ionization masking effects from the presence of a complex mixture including 2,3,4,5-tetrabromopyrrole, which was observed to co-elute with 3,4-dibromopyrrole-2,5-dione under these chromatographic conditions. In addition to numerous halogenated metabolites, we also were able to observe other unique metabolites produced by members of Clade IV (versus other *Pseudoalteromonas* clades), when using PCA to compare metabolomes generated with +HRESI data (n = 1552 chemical features; Figures S31, S32 and S33, Tables S4, S5 and S7, Supporting Information), although we did not follow up with a complete characterization of these chemical features to avoid over-interpretation of these data. Strain A746 was distinguished by a group of 177 chemical features (Figures S31 and S32, Table S4 Supporting Information) whereas other members of Clade IV (strains A575, A574, B149) were more easily distinguished by a group of 56 chemical features (Figure S31 and S33, Table S5, Supporting Information). Although we did not seek to fully annotate chemical features detected in +HRESI, these data also indicate (along with –HRESI results) that members of Clade IV do possess a characteristic exo-metabolome.

Screening features detected with our untargeted metabolomics approach (both –HRES and +HRES) against an in-house database containing previously reported *Pseudoalteromonas* metabolites indicated isolates in our collection potentially produce molecules previously described for *Pseudoalteromonas* spp. Molecular features annotated in +/-HRESI mode data with isotopic distribution patterns and predicted formulae matching these dereplicated compounds are shown in Figure 3 and Tables S8 and S9, Supporting Information. A potential annotation for the antibiotic, 2-*n*-heptyl-4-quinolinol,⁴⁰ known to also influence bacterium-phytoplankton interactions,²³ was exclusively found within members of Clade IV in both +HRESI and –HRESI data sets (Figure 3). Previous work indicated bacterial production of 2-*n*-heptyl-4-quinolinol is surface-dependent, and bacteria are able to generate localized zones of concentrated 2-*n*-heptyl-4-quinolinol on sinking or suspended particles without loss of this antibiotic to the surrounding seawater.²³ It would be interesting to know if 3,4-dibromopyrrole-2,5-dione has an overlapping accumulation profile as the putative 2-*n*-heptyl-4-quinolinol ion in isolate A757. Moreover, features matching the predicted molecular ions for the antibiotic diketopiperazine, cyclo-(*L*-phenylalanyl-4*R*-hydroxyl-*L*-proline)³⁷ and the antifungal, isatin (1*H*-indole-2,3-dione)⁴¹ were also noted in many isolates in both ionization modes (Figure 3A & B). Clade IV may also exclusively produce the antibiotic, 2-*n*-pentyl-quinolinol⁴⁰ (Figure 3A). Additional annotations for molecular ion matches from our database include: the cryptic metabolite, 3-formyl-*L*-tyrosine-*L*-threonine dipeptide,⁴² three isomers of indole-3-carboxaldehyde,⁴⁰ three isomers of the antibiotic, *p*-hydroxybenzaldehyde⁴⁰ and two isomers of antibiotic, *p*-hydroxybenzoic acid,⁴³ all of which were expressed by the majority of *Pseudoalteromonas* isolates in our collection

(Figure 3A). Molecular features found only in a single isolate include those matching to the ions for the antifungal polyketide, alteramide B⁴⁴ from A757; the antistaphylococcal/antivibrio compound, indolmycin⁴⁵ and Gram-negative antibiotic, korormicin 1b⁴⁶ from A345; and the diketopiperazine, cyclo-(*D*-pipecolinyl-*L*-isoleucine)⁴⁷ from A198 (data not shown). Molecular ions matching the antibiotic diketopiperazine cyclo-(*L*-pipecolinyl-*L*-phenylalanine)³⁷ was found in A474 and A454, while features matching the calculated [M+H]⁺ ions of antibiotic, pseudomonic acid C⁴⁸ and the cytotoxic carboline alkaloid, norharman (9*H*-pyrido[3,4-*b*]indole)⁴⁹ appeared to be expressed in the majority of our isolates, with a few exceptions (Figure 3B). Future work will be able to discern the true identities of these chemical features, as several isomers are possible for each of the detected ions in our analysis. Regardless, chemophylogenetic analysis indicated that some compounds were i) characteristic of a particular isolate, ii) clade specific (e.g., halogenated species, 2-*n*-heptyl-4-quinolinol, and 2-*n*-pentyl-quinolinol) or iii) ubiquitous in the majority of isolates.

Increasingly, non-photosynthetic Gram-negative bacteria (NPGNB) are being recognized as the true source of pharmaceutically-relevant molecules from marine macroorganisms;²⁹ however, the difficulty in culturing marine-derived strains to sufficient quantities has likely hampered intense bio-prospecting efforts.²⁹ Indeed, the majority (86%) of our marine isolates found to have MDR reversal activity in our initial screening efforts fall within the NPGNB group, and have been isolated from both abiotic and biotic surfaces (Table S1, Supporting Information). The cosmopolitan marine genus *Pseudoalteromonas* (class Gammaproteobacteria), which constitute 0.5-6% of bacterial species globally,⁵⁰ has been found in seawater, marine sediments and epiphytically

associated with marine eukaryotes, and has been a prolific source of brominated compounds,^{37, 51, 52} including pentabromopseudilin,³⁶ the first marine microbial natural product to be described.^{53, 54} Compounds isolated from this genus function in multiple ecological roles including their involvement in chemical protection, settlement, germination and metamorphosis of marine invertebrate and algal species, as well as more commercial uses as antifoulants, antibacterial, antifungal and cytotoxic agents.^{37, 52, 55} Recent genome mining work has uncovered the biosynthetic pathways responsible for brominated pyrrole/phenol biosynthesis (*bmp*)³⁸ indole derivatives, siderophores, polyketides, homoserine lactones, peptides (both ribosomal and non-ribosomal origin) and hybrid molecules,⁵⁶ which likely represent just the tip of the iceberg, as the number of pathways encoded in *Pseudoalteromonas* genomes eclipses the fraction of molecules identified thus far.⁵⁵

For marine bacteria, including many antibiotic producing *Pseudoalteromonas* spp.,^{37, 57} a viable strategy by these organisms may be to secrete an EPI to enhance their own antibiotic effectiveness. Previous research suggests the dominance and enriched diversity⁵¹ of *Pseudoalteromonas* spp. in biofilms could be attributed to their ability to rapidly form microcolonies and produce extracellular antibacterial compounds.^{36, 58} The production of EPIs targeting RND pumps and other MDR pumps may effectively disable the antibiotic resistance mechanisms of competitors allowing *Pseudoalteromonas* strains to colonize and persist in biofilms. This chemical strategy of using EPIs to enhance antibiotic potency is not novel in nature; rather it has been previously described in plants mounting an attack against surface-associated Gram-positive bacteria.⁵⁹ A blastp search of all 54 *Pseudoalteromonas* spp. genomes available in the IMG database

(<https://img.jgi.doe.gov/cgi-bin/w/main.cgi>) showed amino acid identities to the inner membrane RND transporters as high as 71% for AcrB, 65% for MexB and 52% for MexY. Efflux pumps with homology to AcrB and MexB are known to be functional in *Vibrio parahaemolyticus* inhabiting brackish saltwater, for example, where the VmeAB efflux pumps have been shown *in vitro* to exhibit bile salt and antibiotic resistance phenotypes.⁶⁰ Although it is unclear what role, if any, the putative RND-type homologues present in *Pseudoalteromonas* spp. may play, it is possible that they could be aiding in moderating allelopathic interactions as was found with antibiotic resistance and production in *Vibrio* spp. bacteria.⁶¹ Considering efflux pump inhibitors as antibiotic adjuvants among natural populations could add a new dimension to our understanding of competition between microbial populations. Our data show that *Pseudoalteromonas* strains indeed display niche specificity in regards to halogenated metabolite production, and further bioprospecting efforts could benefit from a focus on environments - such as biofilms, to enhance discovery of new antibiotic and EPI therapeutics.

EXPERIMENTAL SECTION

General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra (1D ¹H and ¹³C) were recorded on a Bruker Avance III 300 MHz spectrometer in DMSO-*d*₆ with the solvent (δ H at 2.54, δ C at 39.5) used as an internal standard. Chemical shifts are given as δ (ppm). HPLC-MS experiments were accomplished using an Agilent Technologies 6230 ToF with Dual Agilent Jet Stream Electrospray Ionization source, equipped with an Agilent 1260 Infinity series HPLC with a Phenomenex Kinetex™ 2.6 μ m, C18, 100 Å, LC column (150 x 2.1 mm) as the stationary phase. All HPLC-MS experiments used a flow rate of 0.2 mL/min. This instrument was also equipped with

Agilent Mass Hunter Workstation version B0.4.00 software. Mass spectra peak picking and alignment software MZmine 2.11⁶² was used for processing mass spectra before principal component analysis with the PLS Toolbox in Matlab version 8.2.0.7. EPI isolation was accomplished using vacuum liquid chromatography (10 x 5 cm) with Silica gel, pore size 60 Å, particle size 40-75 µm (Sigma-Aldrich, St. Louis, MO, USA). All solvents used throughout the project were OPTIMA grade (Fisher Scientific). Semi-preparative HPLC was carried out on an Agilent 1200 series equipped with an autosampler, diode array detector, quaternary pump, and 96-well plate fraction collector with a Phenomenex Luna® 5 µm C18(2), 100 Å, LC column (250 x 10 mm) as the stationary phase. All semi-preparative HPLC experiments used a flow rate of 4 mL/min. Authentic standards of 2,3,4,5-tetrabromopyrrole (Cat. L165042) and 3,4-dibromopyrrole-2,5-dione (Cat. 553603) were purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) for use in activity assays.

Bacteria Culture and Chemical Library Production. Currently the Mincer Laboratory maintains over 2000 unique marine microbial isolates for chemical exploration. All pure cultures are cryopreserved in 10% sterile DMSO and stored at -85°C. A “starter” culture was prepared by inoculating 100 µL of frozen culture in 6 mL of TSW media (1 g tryptone in 1 L of 75:25 seawater/MilliQ water) and incubated at 23°C, 100 rpm for 3 d. In general, 1.5 L of TSY media (1 g tryptone, 1 g yeast extract, 75% seawater) is inoculated with 1.5 mL of “starter” culture and grown at 100 rpm for 8 d at 23°C. Twenty four hours before culture filtration (Day 7), 20 mL of 1:1 mixture of sterile, washed Amberlite® XAD-7 and XAD-16 resin was added to the cultures. On the eighth day, the resin was filtered from the bacterial culture under vacuum, desalted by

rinsing with MilliQ water, and allowed to dry overnight at room temperature. Metabolites were eluted from the resin first in 100 mL of (1:1) MeOH:DCM, followed by 100 mL of MeOH. This crude organic extract was then dried under vacuum centrifugation (ThermoSavant). Dried extracts were subsequently resuspended in DMSO at 100 mg/mL and stored at -85°C until further testing in bacterial susceptibility assays.

Bacterial Susceptibility Determinations. Whole-cell assays were used to search for efflux pump inhibitors for three archetype RND efflux pumps (AcrAB-TolC, MexAB-OprM, and MexXY-OprM) that are known to contribute to antibiotic resistance in Enterobacteriaceae and *P. aeruginosa* clinical isolates. *Escherichia coli* strains engineered to overexpress RND transporters were generous gifts from Dr. C. Elkins (U.S.F.D.A.)³⁰ and Dr. Y. Matsumoto (Osaka University).⁶³ Isolates used for screening include AG102 (derived from AG100; *E. coli* K-12), an isolate that overexpresses AcrAB-TolC efflux system due to a mutation in the MarR (marR1) which increases the expression of MarA, a global regulator, which in turn results in the overexpression of AcrAB-TolC efflux system;³⁰ and two *E. coli* MG1655 deletion mutants (Δ acrB Δ tolC) that have been transformed with the plasmid carrying genes *mexAB-oprM* (MG1655 Δ BC/pABM) and *mexXY-oprM* (MG1655 Δ BC/pXYM) from *P. aeruginosa*.⁶³ *E. coli* strains harboring plasmids were always cultured in a medium containing 100 μ g/mL ampicillin and 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG).

Minimum inhibitory concentrations (MICs, defined as the lowest concentration that will inhibit detectable culture growth) of the antibiotics (chloramphenicol and erythromycin, both of which are substrates of RND pumps)^{30, 64-67} were determined using a twofold standard microdilution method in Muller-Hinton broth (MHB) in microtiter

plates as defined by the National Committee for Clinical Laboratory Standards⁶⁸ for each of the three *E. coli* strains. Bacterial susceptibility of crude extracts were tested in duplicate at 1 mg/mL and determined by rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay in 96-well microtiter plates in a final volume of 200 μ L as described in Kuete et al. 2010.⁶⁹ The INT assay evaluates if crude extracts have an MDR reversal effect (so called ‘MIC/4’, defined as reducing by at least fourfold the antibiotic MICs) by examining the growth of our test *E. coli* strains in the presence of crude extract and either chloramphenicol or erythromycin at concentrations at $\frac{1}{4}$ their MICs. If the crude extract is capable of potentiating the concentration of antibiotic needed to inhibit the growth of one or more test strains of MDR *E. coli*, these initial “hits” were then prioritized for further testing (in a serial dilution series) in the presence and absence of antibiotic via the INT assay to establish if EPI-like activity was present. Wells containing MHB, inoculum and DMSO at a final concentration of 1% served as a negative control. Phenylalanyl arginyl β -naphthylamide (PA β N, general RND pump inhibitor) was used as a positive control at 30 μ g/mL in INT assays.

Culture Production Scale-up, Bioassay Guided Fractionation, and Chemical Analysis. Marine isolate A757 (GenBank KM596702), determined to be most closely related to *P. piscicida* by 16S rRNA sequence comparison, was targeted for scaled-up regrowth to obtain additional material for chemical identification of the putative EPI compound. A starter culture of A757 was used to inoculate eleven, 1.5 L Fernbach flasks for a total of 16.5 L of culture medium that was processed as described in “Bacteria Culture and Chemical Library Production”, yielding a total of 1729.7 mg of crude organic extract. At each subsequent fractionation step an aliquot of material was

resuspended in DMSO and assayed in the INT assay against MG1655 Δ BC/pXYM to confirm retention of MDR reversal activity. A total of 1.7 g of crude extract was applied to a silica gel column and eluted with a step-gradient of 100% isooctane, 4:1 isooctane/EtOAc, 3:2 isooctane/EtOAc, 2:3 isooctane/EtOAc, 1:4 isooctane/EtOAc, 100% EtOAc, 1:1 EtOAc/MeOH, and 100% MeOH, yielding eight fractions. Active constituents eluted with 3:2 isooctane/EtOAc and 2:3 isooctane/EtOAc, were combined, and chromatographed on a semipreparative HPLC using a gradient of ACN (0.1% formic acid) and H₂O (0.1% formic acid). Chromatography methods were as follows: hold at 5% ACN for 5 mins, ramp to 40% ACN over 5 min, hold at 40% ACN for 2 mins, ramp to 95% ACN over 5 min, hold at 95% ACN for 7 min, ramp down to 5% ACN and hold for 4 min. Active constituents eluted with 95:5 ACN/H₂O at a flow rate of 4 mL/min over 28 min into a 96 deep-well plate. Two wells on the 96 deep-well plate responsible for the activity were combined and subject a second round of semipreparative HPLC (ramp from 55-75% ACN over 20 min; ramp to 95% ACN; hold at 95% ACN for 6 min; ramp to 55% ACN and hold for 2 min, 4 mL/min flow rate), with active constituents eluting with 70:30 ACN/H₂O into a 96 deep-well plate resulting in activity spread over three wells containing 14.4 mg of material. LC-MS analysis with acidified solvents (0.1% formic acid) was performed on the active fraction with a solvent gradient of 6:94 MeOH/H₂O ramping to 8:92 MeOH/H₂O over 25 mins, at a flow rate of 0.2 mL/min, which led to the identification of 3,4-dibromopyrrole-2,5-dione, the major component of the fraction. NMR spectra of 3,4-dibromopyrrole-2,5-dione from A757 and an authentic standard are available in Figures S2-5 in Supporting Information.

Checker board Assay. To assess the interaction between different classes of antibiotics and 3,4-dibromopyrrole-2,5-dione, we performed standard checkerboard assays in which the MICs of antibiotics were determined in the presence of different concentrations of 3,4-dibromopyrrole-2,5-dione. Standard checkerboard titration microtiter plate assays were performed as described in^{65, 70} to determine the fractional inhibitory concentrations (FICs) of 3,4-dibromopyrrole-2,5-dione against various antibiotics (chloramphenicol, ciprofloxacin, erythromycin, kanamycin, levofloxacin, oxacillin, piperacillin, and tetracycline) for all three *E. coli* strains.

Hoechst Accumulation and Efflux Assay. To truly be considered an EPI, the compound must increase the level of accumulation and decrease the level of extrusion of efflux pump substrates. The fluorescent DNA-binding dye Hoechst 33342 (H33342) is a substrate for RND pumps, is easily detected in the cell, can freely permeate the outer membrane, and can act as a reporter -- all allowing for quantification of transport across living cells.⁷¹ Efflux competent cells extrude H33342 and accumulate dye at a relatively slow rate, resulting in low levels of background fluorescence. Conversely, efflux-defective cells (e.g., presence of EPI) accumulate intracellular levels of H33342 at a higher rate, resulting in fluorescence retention. Wild-type *E. coli* K-12 strain, AG100, and AG100A, the *ΔacrAB* mutant, were a generous gift from Dr. M. Viveiros (Universidade Nova de Lisboa, Portugal) and used in accumulation and efflux assays.^{72, 73} Experiments were performed in microtiter plate format as described in^{74, 75} using H33342 as described in⁷¹ at a concentration that does not affect the growth of *E. coli* strains. All wells were performed in duplicate. Heat-inactivated bacteria were used as a positive control to assess maximal dye accumulation (data not shown).

For accumulation assays, in efflux competent cells like AG100, H33342 would be extruded resulting in low levels of background fluorescence, while the presence of a putative EPI would cause intracellular H33342 accumulation. Moreover, AG100A, engineered to lack efflux pumps, was used to confirm that 3,4-dibromopyrrole-2,5-dione is indeed targeting efflux pumps, and that MDR reversal is limited to efflux pump over-expressing test *E. coli* strains. Specifically, accumulation of H33342 in AG100A cells would be unaffected by the presence of 3,4-dibromopyrrole-2,5-dione. Briefly, AG100 and AG100A were grown in Luria Broth (LB) at 37°C and 200rpm until an OD₆₀₀ of 1.0 then pelleted at 3000 rpm for 15 min, washed twice with phosphate buffered saline (PBS), and diluted in PBS (without glucose) to OD₆₀₀ 0.3. The following conditions were used to achieve a minimal accumulation of H33342 in AG100 and AG100A cells: the use of 2.5 µM H33342 in the presence of 22 mM glucose at 37°C. PAβN (15.6 µg/mL) was used as a positive control. Fluorescence values for wells containing media and compounds only were subtracted from those containing bacteria to control for any background fluorescence due to the presence of test compounds. Bacteria in PBS without H33342 + glucose + 3,4-dibromopyrrole-2,5-dione were also used as a control (data not shown). Bacterial cells were allowed to incubate for ~30 min until H33342 accumulation stabilized, after which 3,4-dibromopyrrole-2,5-dione (concentrations ranging from 0.06 – 64 µg/mL) and PAβN (15.6 µg/mL) were added. Accumulation of H33342 was continuously monitored (Ex350nm/Em460nm) for another 60 min using a SpectraMax M2.

For efflux assays, a time-dependent decrease in fluorescence of H33342-loaded AG100 would be observed only when efflux is active, while the presence of a putative EPI would

cause the retention of H33342 over time. Briefly, AG100 cells were grown, pelleted and washed as described above, then “loaded” with H33342 (2.5 μ M) and either PA β N (15.6 μ g/mL) or 3,4-dibromopyrrole-2,5-dione (concentrations ranging from 1 – 64 μ g/mL) under conditions that favor maximal accumulation (no glucose, 25°C). When maximum accumulation was reached (after 60 min), bacteria were pelleted at 3000 rpm for 3 min, resuspended in ice-cold PBS, aliquoted into the microtiter plate, and exposed to corresponding concentrations of either PA β N or 3,4-dibromopyrrole-2,5-dione, with and without glucose at 37°C. H33342 efflux was continuously monitored by fluorescence at (Ex 350 nm/Em 460 nm) for an additional 30 min. Values from the “no glucose” control wells were subtracted from the values obtained from wells containing glucose. To obtain a comparative analysis of the efflux, the fluorescence data of the H33342-loaded cells were normalized to 1, thereby establishing a maximum fluorescence value.

Statistical comparisons of H33342 accumulation assay data were performed by combining fluorescence measurements from the final four time points (period where accumulation/fluorescence has stabilized) and comparing fluorescence among treatments using a 1-way ANOVA with a Dunnett’s post-test to determine statistical differences between treatments and the Hoechst control (Graphpad Prism 6.05). To calculate the H33342 efflux IC₅₀ concentration of 3,4-dibromopyrrole-2,5-dione, final fluorescence measurements (from final four data points, as described above), were used to plot efflux versus EPI concentration. The line was then fitted to a sigmoidal curve and IC₅₀ was calculated in Graphpad.

Chemical profiling with mass spectrometry. Exuded secondary metabolites produced by *Pseudoalteromonas* spp. extracts (n = 36 individual strain extracts) were diluted to 5

mg/mL in DMSO for untargeted metabolomic fingerprint analysis. Standards of 2,3,4,5-tetrabromopyrrole (in a standard curve spanning two orders of magnitude) were used to determine the retention time and limit of detection (calculated as $3 \times \text{S.D.} / \text{slope}$ of regressed standard curve). Extracts were then profiled (5 μL injections) by reversed-phase HPLC/MS ToF, using MeOH (0.1% formic acid) and H₂O (0.1% formic acid). Chromatography methods were as follows: hold at 5% MeOH for 5 min, ramp to 40% MeOH over 5 min and hold for 2 min, ramp to 95% MeOH over 5 min and hold for 3 min. The first 4.5 min of each run was not injected into the mass spectrometer to avoid DMSO contamination, and there was an 8 min column equilibrium time between injections. Column temperature was held at 35°C with a flow rate of 0.2 mL/min.

For profiling, spectra were collected in both positive (+HRESI) and negative (-HRESI) ionization modes in two separate runs (i.e., no polarity switching). Settings were as follows for +HRESI: mass correction ions used were 922.0098 m/z and 121.0509 m/z (injected at source); scanning a 20-3000 m/z range with a scan rate of 1.00; gas temperature and sheath gas temperature at 350°C. Drying gas flow rate was 8 L/min, while sheath gas flow rate was 10 L/min with nebulizer pressure set to 40 psi. Capillary voltage and nozzle voltages were 3500 V and 1000 V, respectively. Fragmentor and skimmer voltages were 135 V and 65 V, respectively. These settings were held for -HRESI except for the mass correction ions used, which were 1033.9881 m/z and 112.9856 m/z in negative mode.

Metabolomics processing and statistical analysis. For metabolomics analysis, individual spectra were exported from Mass Hunter Workstation as .mzData files and imported in MZmine⁷⁶ for preprocessing, as performed in.^{77, 78} This included mass

detection, chromatogram building, peak convolution, deisotoping, retention time normalization, spectral alignment, gap-filling, and duplicate peak filtering. The same algorithms and settings were used for both +HRESI and -HRESI data, except that minimal peak thresholds were set to 5000 ion counts for -HRESI and 10,000 ion counts for +HRESI data. After processing, a list of chemical features (m/z -retention time pairs) was filtered to only include those features greater than an order of magnitude more concentrated in a *Pseudoalteromonas* sample than in the media blank. For +HRESI data, all features detected between 18.99 – 23.0 min were also removed due to contamination from polyethylene glycol during those retention times. Filtered datasets were then imported into Matlab and autoscaled⁷⁹ before principal component analysis (PCA) using the PLS Toolbox⁸⁰, while +HRESI and -HRESI data were analyzed separately. PCA was used to visualize differences among the exuded metabolite profiles of the *Pseudoalteromonas* strains. The loadings for particular principal components were examined to determine which suites of chemical features were likely to be differentially expressed³⁵ in different clades of *Pseudoalteromonas*, and in particular Clade IV. Mass Hunter Workstation (Qualitative Analysis) was used to confirm the presence of particular chemical features in raw (unprocessed) mass spectra, and to confirm isotopic distributions of any halogenated chemical features that were important in distinguishing *Pseudoalteromonas* Clade IV in the PCA. To screen for the presence of known *Pseudoalteromonas*-specific metabolites in our extracts, a literature search was performed and previously described compounds (including their names, monoisotopic mass, and molecular formulae) were compiled into our in-house “*Pseudoalteromonas* database.” The feature list generated in MZmine (see above) was then searched against

this database (assuming a [M-H]⁻ adduct for -HRESI or [M+H]⁺ adduct for +HRESI) and with a mass error threshold of ≤ 5.0 ppm.

Phylogenetic Analysis. The evolutionary history was inferred using the Neighbor-Joining method⁸¹ and the optimal tree is shown for topology. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)⁸² are shown next to the branches for Neighbor Joining (left) and Maximum Likelihood (right). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model.⁸³ Initial tree(s) for the heuristic search of the Maximum Likelihood tree nodes were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method⁸⁴ and are in the units of the number of base substitutions per site. The analysis involved 73 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 399 phylogenetically informative positions in the final dataset. Evolutionary analyses were conducted in MEGA6.⁸⁵

ASSOCIATED CONTENT

Supporting Information.

Chemical and physical data including NMR spectra; MS-ToF data; HPLC data; additional metabolomics analyses including PCA scores and loadings plots, tables of distinguishing chemical ions and compounds, mass spectra of brominated compounds,

and database annotation are reported in Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes.

Accession numbers for *Pseudoalteromonas* sp. strains identified in our screening efforts are deposited in GenBank (KM596668 through KM596703), and metadata describing their coordinates of collection, date, sample description, and strain collection number can be found in Table S1 in Supporting Information.

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Notes

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ABBREVIATIONS

CIP, ciprofloxacin; CHL, chloramphenicol; EPI, efflux pump inhibitor; ERY, erythromycin; FICI, fractional inhibitory concentration index; INT, *p*-Iodonitrotetrazolium chloride; KAN, kanamycin; LEV, levofloxacin; MIC, minimum inhibitory concentration; MDR, multidrug resistance; OXA, oxacillin; PA β N, phenylalanyl arginyl β -naphthylamide; PIP, piperacillin; RND, Resistance Nodulation Cell Division; TET, tetracycline.

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Figure 2. Phylogenetic and exo-metabolomic comparison of *Pseudoalteromonas* sp. (A) Neighbor-Joining dendrogram displaying the evolutionary history of the 16S rRNA gene sequences of the 36 *Pseudoalteromonas* spp. featured in this study. Topology of the tree was tested by 1000 bootstrap replicates and scores are displayed for Neighbor-Joining (top) and Maximum Likelihood (bottom) at each node. Scores under 50% are not shown. (B) PCA Scores plot of exo-metabolomes (-HRESI) of, 36 different *Pseudoalteromonas* strains representing seven clades (I-VII). The first principal component (PC1) describes 20.7% of the variance while the second principal component (PC2) describes 16.2% of total variance. Dashed line indicates 95% confidence interval. (C) Heat map displaying relative abundances of brominated metabolites produced exclusively by *Pseudoalteromonas* Clade IV (strains A757, A754, A746, and B149). Colored bar rank peak areas of the major brominated isotope of each metabolite. Column headings display each ion (m/z) with retention time (RT) for each ion in minutes. Peak areas were calculated in MZmine. ND = non-detect.

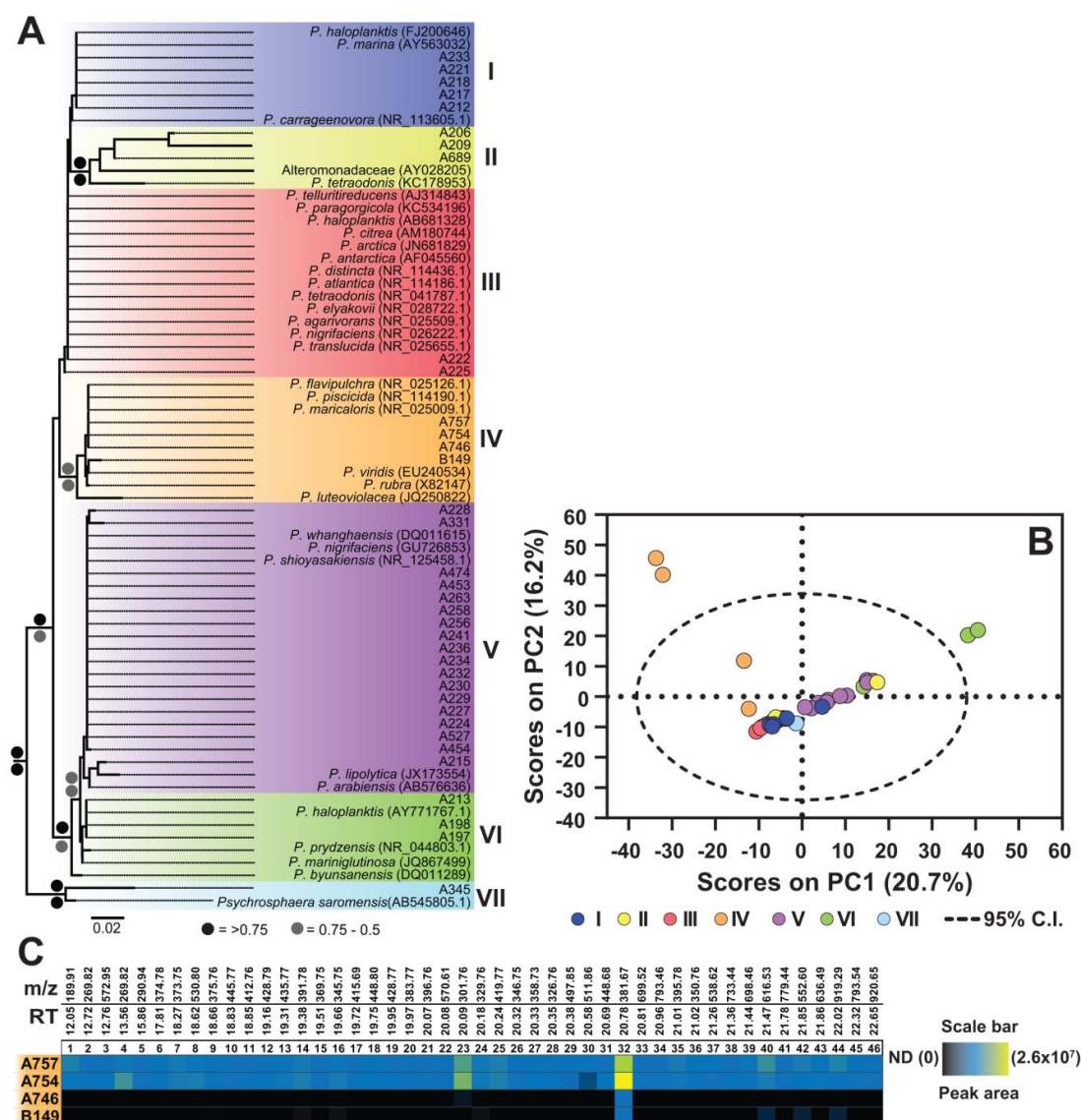
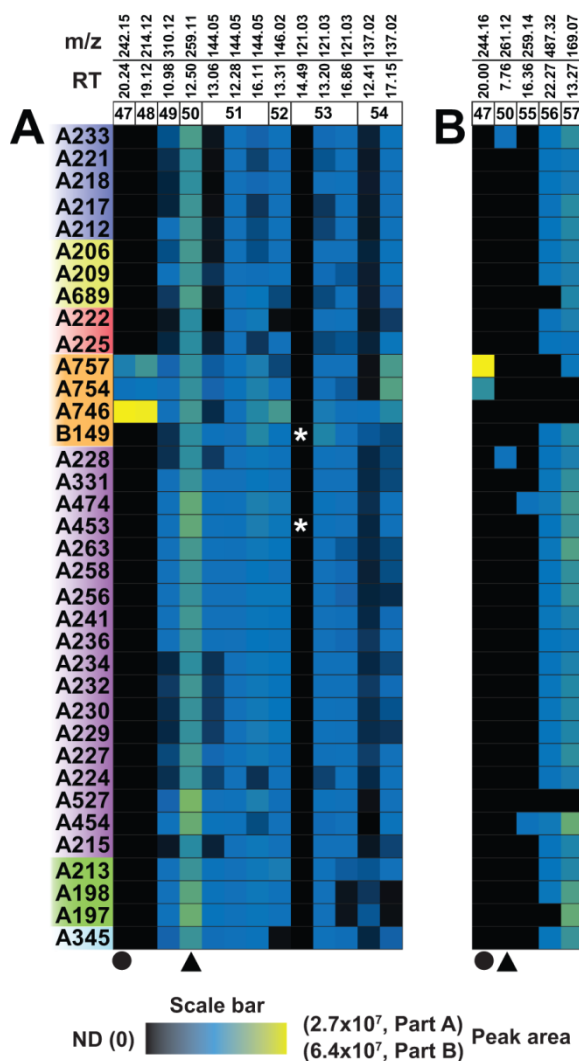


Figure 3. Heat map displaying relative abundances of inferred compounds produced by *Pseudoalteromonas* isolates from (A) –HRESI and (B) +HRESI mode data. Colored bars indicate peak areas of each ion (m/z) with the corresponding retention time (RT) in minutes. Preliminary compound identification is as follows: 2-*n*-heptyl-4-quinolinol (47), 2-*n*-pentyl-quinolinol (48), 3-formyl-*L*-tyrosine-*L*-threonine dipeptide (49), cyclo-(*L*-phenylalanyl-4*R*-hydroxyl-*L*-proline) (50), three isomers of indole-3-carboxaldehyde (51), isatin (1*H*-indole-2,3-dione) (52), three isomers of *p*-hydroxybenzaldehyde (53), two isomers of *p*-hydroxybenzoic acid (54), cyclo-(*L*-pipecolinyl-*L*-phenylalanine) (55), pseudomonic acid C (56), 9*H*-pyrido[3,4-*b*]indole (57). Asterisks indicate a low abundance ion (121.03, RT = 14.49 min) in isolate B149 (peak area = 5129) and isolate A453 (peak area = 3589). The presence of two inferred compounds (●) 2-*n*-heptyl-4-quinolinol (46) and (▲) cyclo-(*L*-phenylalanyl-4*R*-hydroxyl-*L*-proline) (49) were present in both –HRESI (A) and +HRESI(B) heat maps. Peak areas were calculated in MZmine. ND = non-detect.



CHAPTER 3

This manuscript is formatted for publication in Marine Drugs. The formatting follows the guidelines set forth by the journal.

A Marine Bacteria-Produced Benzisoxazole with Antibacterial Activity Against Multi-Drug Resistant *Acinetobacter baumannii*

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Abstract

The emergence of multidrug resistant (MDR) pathogenic bacteria represents a serious and growing threat to national healthcare systems. Most pressing is an immediate need for the development of novel antibacterial agents to treat Gram-negative MDR infections, including the opportunistic, hospital-derived pathogen, *Acinetobacter baumannii*. Herein we report a naturally occurring 1,2-benzisoxazole with minimum inhibitory concentrations as low as 6.25 µg/mL against clinical strains of MDR *A. baumannii*. This molecule represents a new chemotype for antibacterial agents against *A. baumannii* and is easily accessed in two steps through total synthesis. Structural analogs suggest that the natural compound may already be optimized for activity against this pathogen. Further studies to advance the development of this 1,2-benzisoxazole as an antibiotic for treatment of MDR *A. baumannii* infections are strongly warranted.

Keywords: multidrug resistant bacteria, *Acinetobacter baumannii*, benzisoxazole, antibacterial

1. Introduction

Acinetobacter baumannii is a non-fermenting, Gram-negative bacterium commonly implicated in nosocomial infections such as wound infections, sepsis, and ventilator associated pneumonia.[1] The World Health Organization recognizes antibiotic resistance as one of the three greatest threats to human health, and recently classified carbapenem-resistant *A. baumannii* as one of the most urgent threats, calling for a renewed investment in antibiotic development.[2] *A. baumannii* tenacity in hospitals is enhanced both by its ability to develop antibiotic resistance and capacity to survive on surfaces, including skin, for several days.[3] *A. baumannii* can also spread via aerosolization, promoting this pathogen's ability to easily colonize new environments.[4-6] *A. baumannii* is a member of the 'ESKAPE' pathogens [7] and has garnered significant attention due to its naturally occurring resistance to the most commonly used antibiotics.[8] Alarming, intensive care unit patients contracting *A. baumannii* infections have a high mortality rate (>50%), complicated by the fact that new pan-drug resistant (PR) strains of *A. baumannii* with resistance to all treatment options have now emerged.[1,8-10] Carbapenems, typically in combination with other antimicrobial agents (e.g. colistin) are a commonly utilized treatment option for MDR *A. baumannii* but it is estimated that more than half of MDR strains are now carbapenem-resistant.[1,11,12] Currently, the polypeptide antibiotic colistin is the primary method of treatment for patients with carbapenem-resistant *A. baumannii*; however, this therapy has significant toxicological and dosing concerns.[1,12] With the lack of new antibiotics in the drug discovery pipeline to treat Gram-negative infections, coupled with accelerated evolution

of antibiotic resistance, it is imperative that new antibacterial drugs for treating *A. baumannii* infections be developed.[13]

Most antibacterial drugs are products of microbial biosynthesis and derivatives thereof.[14] Herein we report the identification of a 1,2-benzisoxazole antibiotic produced by a marine bacterium identified as a *Bradyrhizobium denitrificans*. A series of synthetically prepared analogs define key structural features for the antibacterial effects against *A. baumannii*. This molecule represents a new lead structure for antibacterial drug development against clinical isolates of *A. baumannii* that possess MDR phenotypes.

2. Results

Exudate extract from *Bradyrhizobium denitrificans* (Isolate B158) was initially screened in the *p*-iodonitrotetrazolium chloride (INT) assay to assess MDR reversal potential.[15] Phylogenetic analysis by 16S rRNA gene sequence comparison indicated that isolate B158 was most closely related to *Bradyrhizobium denitrificans*. Bioassay-guided fractionation of extract generated from B158 microbial culture resulted in the isolation of **1** and two other known compounds (lumichrome and (1*H*-indol-3-yl) oxoacetamide; see **11** and **12** in Supplementary Materials) [16,17] with varying degrees of bioactivity.

Compound **1** was isolated as an amorphous white powder. An $[M-H]^-$ ion of 150.0204 using HRESIMS indicated a molecular formula of $C_7H_5NO_3$. 1H -NMR resonances at δ_H 7.49, 6.75, and 6.67 as well as the molecular formula suggested **1** to be 3,6-dihydroxy-benzisoxazole.[18] Because of limited quantity of the natural product, 3,6-dihydroxy-benzisoxazole was synthesized (**Scheme 1**) and determined to be identical to the natural product by NMR (**Table 1**) and LC/MS analysis (**Figure S2**).[18]

Scheme 1: Synthesis of 3,6-dihydroxy-benzisoxazole (**1**). *a* – H₂NOH:HCl/KOH in Dioxane/H₂O; *b* – CDI/Et₃N/THF reflux.

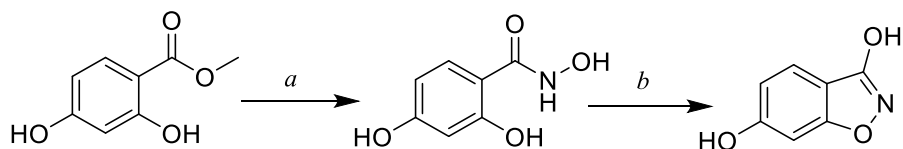


Table 1: Comparison of ¹H and ¹³C NMR data of **1**, synthetic 3,6-dihydroxy-1,2-benzisoxazole, and published 3,6-dihydroxy-1,2-benzisoxazole.[18] Chemical shifts are referenced to residual MeOH in CD₃OD at δ_H 3.31 and δ_C 49.0.

	Synthetic 3,6-dihydroxy-1,2-benzisoxazole (1)		Published 1	Isolated 1
position	δ_C , type	δ_H , (J in Hz)	δ_C , type	δ_H , (J in Hz)
3	167.6, C		167.4, C	
3a	108.2, C		108.1, C	
4	123.6, CH	7.50, d (8.6)	123.6, CH	7.49, d (8.6)
5	114.5, CH	6.77, dd (8.6, 1.9)	114.5, CH	6.75, d (8.6)
6	163.2, C		163.1, C	
7	96.2, CH	6.70, d (1.9)	96.2, CH	6.67, s
7a	166.9, C		166.8, C	

Compound **1** was previously reported to possess growth inhibitory effects against Gram-negative bacteria, but not Gram-positive pathogens.[19] Because this prior testing was completed more than 30 years ago, we screened **1** for antibacterial activity against contemporary and MDR Gram-negative bacteria (data not shown) including two beta-lactamase producing *E. coli*, a carbapenemase producing *Klebsiella pneumoniae*, and an MDR *A. baumannii* (L1051). Of the strains included, **1** showed promising activity (MIC of 8-16 $\mu\text{g/mL}$) only against the *A. baumannii* isolate, which merited follow up testing.

A panel of four unique *A. baumannii* strains was compiled to assess the effectiveness of **1** in clinically relevant, antibiotic resistant strains. The panel included one strong biofilm forming strains (L1184), one moderate biofilm forming strain (L1227), one weak biofilm forming strain (L1222), and one biofilm non-forming strain (L1051). Two strains (L1222 and L1051) were classified as multi-drug resistant (MDR) organisms by having non-susceptibility to one or more agents in three or more antimicrobial categories using previously published guidelines.[20] Minimum inhibitory concentrations (MICs) were determined for **1** (**Table 2**) to be in the range of 6.25 µg/mL to 50 µg/mL with the most potent antibacterial effects against the MDR strains L1222 and L1051 (6.25 and 12.5 µg/mL, respectively). The strong biofilm forming strains L1227 and L1184 were not as sensitive to **1** with MICs of 25 and 50 µg/mL, respectively. All minimum bactericidal concentrations (MBCs) for **1** were >100 µg/mL.

To further understand the structural requirements necessary for the antibacterial effects of **1** against *A. baumannii*, as well as to potentially improve upon the potency of **1**, we synthesized and purchased analogs to perform a structure activity relationship (SAR) study (**Figure 1**). None of the analogs (**2-10**) demonstrated increased potency against the panel of *A. baumannii* strains (**Table 2**). However, structural requirements for optimal potency were revealed. Replacement of the hydroxyl substituent at C6 (as in **2-6**) displayed marked decreases in potency. Based on these data, a hydrogen bond donor substituent at C6 appears to be required for antibacterial effects, with hydroxyl (**1**) as the preferred substituent over amino (**5**). Though the C6 methoxyl group (**6**) did not completely abolish activity, the dramatic decrease in potency concludes that alkyl modifications to the hydroxyl group are unlikely to improve potency in this

pharmacophore. Because of the preference of the hydroxyl substituent at the C6 position, it was also investigated whether an additional hydroxyl group might improve the activity. Surprisingly, a second hydroxyl group at C4 (**7**) abolished the antibacterial activity.

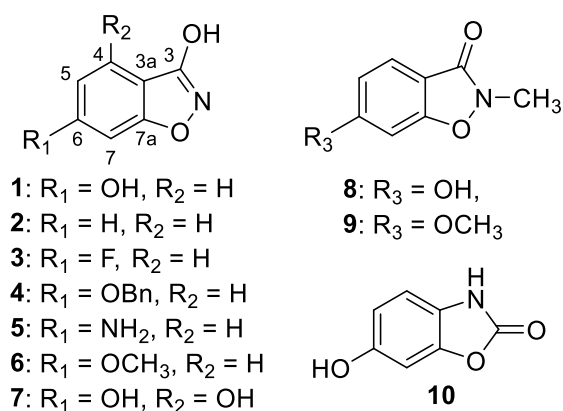
Table 2: MIC values for analog compounds **1-10** against *A. baumannii* strains. Values in bold indicate an inhibition was shown.

A. <i>baumannii</i> Strain	Source	MIC Values ($\mu\text{g/mL}$)									
		1	2	3	4	5	6	7	8	9	10
L1051*	Clinical	12.5	>100	>100	>100	50	>100	>100	>100	>100	>100
L1222*	Blood	6.25	>100	>100	>100	50	100	>100	>100	>100	>100
L1227	Blood	25	>100	>100	>100	50	100	>100	>100	>100	>100
L1184**	Urine	50	>100	>100	>100	50	>100	>100	>100	>100	>100

*Multi-drug resistant organism (MDRO)

**Strong biofilm forming organism

Figure 1: Panel of compounds tested for MICs against *A. baumannii* in SAR study.



In addition to substituents on the benzene ring, the necessity of the isoxazole ring to the pharmacophore was investigated. Methylation of the nitrogen atom (**8** and **9**) led to inactive products; it is noted that this modification leads to loss of conjugation in the hydroxyl-isoxazole ring. Similarly, antibacterial activity was also completely absent for the oxazolone analog (**10**).

3. Discussion

Current therapies for MDR *A. baumannii* are failing. Resistance to the preferred antibiotic class, carbapenems, is rising and many infections are treated with colistin which has serious concerns of neurotoxicity and nephrotoxicity.[1] With a dwindling supply of viable treatments, investigation of new chemical scaffolds with antibacterial effects against *A. baumannii* is critical. 1,2-benzisoxazoles, especially the optimized structure in **1**, represent a new chemotype for the development of therapeutics to treat MDR *A. baumannii* infections.

From the panel of analogs tested, it appears that the structural features in the natural product **1** are necessary components of the pharmacophore. A hydrogen bond donor is necessary at C6, and a hydroxyl is preferred over an amino group. Modifications to the heteroaromatic ring are detrimental when the isoxazole confirmation is not retained. Further optimization of the structure for antibacterial effects against *A. baumannii* would include determining if the 3-position is modifiable, as well as exploring other positions on the benzene ring for substitution.

1,2-benzisoxazoles are bicyclic, heteroaromatic structures found in many approved drugs.[21,22] In general, 1,2-benzisoxazoles are able to cross the blood brain

barrier (BBB) and have been classified as privileged structures for drug design for CNS disorders and other therapeutic areas.[21,22] The heterocyclic ring structure of **1** shares similarities to two clinically used antibiotics: cycloserine and linezolid (and other oxazolidinone antibiotics). Interestingly, cycloserine was inactive against our panel of *A. baumannii* strains (data not shown). Oxazolidinone antibiotics are only effective against Gram-positive pathogens, and match the ring structure of the inactive **10**, further supporting the highly specific nature of the 1,2-benzisoxazole pharmacophore.

In the search for antibacterial compounds for MDR pathogens, it has been shown that ‘old’ antibiotics that have been excluded from the clinic for decades can be useful as alternative treatment options.[23,24] In addition to older clinical antibiotics, there are many antibacterial compounds discovered decades ago that have not been developed, including compound **1** that may be useful against modern MDR pathogens. Compound **1** was previously reported to possess potent growth inhibition of Gram-negative bacteria including *E. coli*, *Proteus* spp., *Salmonella* spp., *Klebsiella pneumoniae*, *Enterobacter* spp., and *Serratia marcescens*, as well as low toxicity with an LD₅₀ of over 1500 mg/kg in mice.[18,19] No inhibition was observed with the Gram-positive bacteria *Staphylococcus aureus*, *Bacillus* spp., *Micrococcus* sp., *Corynebacterium* sp., or the Gram-negative *Pseudomonas* spp.[18,19]

Narrow spectrum of antibacterial drugs are becoming more desirable due to advances in diagnostic tests and changes to the regulatory environment.[25] Resistance is expected to develop more slowly to narrow spectrum antibiotics and collateral damage to the gut microbiome, such as with antibiotic-associated colitis, is less likely.[26] We suggest that further studies advancing the development of 1,2-benzisoxazoles, including

1, as anti- *A. baumannii* antibiotics are warranted. Investigations into its cellular target, *in vivo* efficacy, and further structure activity relationships are vital next steps to improving the outlook for treatment of infections with MDR *A. baumannii*.

4. Materials and Methods

4.1 General Experimental Procedures

NMR experiments were conducted using an Agilent NMRS 500 MHz spectrometer with (CD₃)₂SO (referenced to residual DMSO at δ_{H} 2.50 and δ_{C} 39.5) or CD₃OD (referenced to residual MeOH at δ_{H} 3.31 and δ_{C} 49.0) at 25 °C. High Resolution Electrospray ionization mass spectra (HRESIMS) were acquired using either an AB Sciex TripleTOF 4600 spectrometer in the negative ion mode or Agilent Technologies 6230 ToF with a Dual Jet Stream Electrospray Ionization source, equipped with an Agilent 1260 Infinity series HPLC. Flash chromatography was completed with a Combiflash Rf200 equipped with a 40 g silica gel RediSepRf High Performance Gold column (Teledyne ISCO). HPLC experiments were performed on a Shimadzu Prominence-i LC-2030C system equipped with a diode array detector or an Agilent 1200 series equipped with an autosampler, diode array detector, quaternary pump and 96 well plate fraction collector. Semi-preparative HPLC purifications were accomplished with a Waters XBridge Prep C18 5 μm , 10 \times 250 mm column at 4.5 mL/min. The exact equipment in separation and identification used is specified below. All chemicals and solvents were at least reagent grade and purchased commercially (Sigma Aldrich, St. Louis, MO).

4.2 Bacterial Strains

We examined four clinically diverse strains of *A. baumannii* obtained from patients at Rhode Island Hospital, American Type Culture Collection (ATCC), and the Biodefense and Emerging Infection Research Resources Repository (BEI). Strains depicted various biofilm-forming capabilities (described in Supplementary Materials [27-31]) (non adherent [n=1]; weak [n=1]; moderate [n=1]; strong [n=1]) and resistance patterns, with two **multi-drug resistant organisms** (MDROs) and two non-MDROs. MDRO was defined as organism with non-susceptibility to one agent in three antimicrobial classes, according to previously described definitions.[20] These strains are available in the investigators' laboratory and stored frozen at -80 °C in 20% glycerol.

4.3 *A. baumannii* Susceptibility Testing

Minimum inhibitory concentrations (MICs) of pure compounds against clinical isolates of *A. baumannii* were determined in duplicate by broth microdilution in accordance with Clinical Laboratory Standards Institute (CLSI) standards.[32,33] Due to antibacterial activity of DMSO at high concentrations,[34] our methods were modified to ensure DMSO concentrations do not exceed 2.5%. Plates were subsequently incubated at 37 °C for 24 h. After 24 h, minimum bactericidal concentrations (MBCs) were determined in duplicate for each antimicrobial compound in accordance with CLSI.[32,33]

4.4 Cultivation and Extraction of Marine Bacterial Strain B158

Marine isolate B158 was originally isolated from the oral cavity of the ctenophore, *Mnemiopsis leidyi* from Eel Pond, Woods Hole, MA, U.S.A. in September 2012. The isolate was streaked to purity on TSW (1 g of tryptone in 1 L of 75:25 natural seawater/Milli-Q water) agar plates and cryopreserved in 10% sterile DMSO and stored

at -85 °C. Strain B158 was identified as *B. denitrificans* based on 98% nucleotide sequence identity to available *B. denitrificans* 16S rDNA sequence (Genbank Accession Number MF113387). A “starter” culture was prepared by inoculating 100 µL of cryopreserved culture in 6 mL of TSW media and incubated at 23 °C, 100 rpm for 3 days. A 1.5 L fernbach flask containing TSY media (1 g of tryptone, 1 g of yeast extract in 1 L of 75:25 natural seawater/Milli-Q water) was inoculated with 1.5 mL of “starter” culture and grown at 100 rpm for 8 d at 23 °C. Twenty-four hours before culture filtration (day 7), 20 mL of a 1:1 mixture of sterile, washed Amberlite XAD-7 and XAD-16 resin was added to the cultures. On the eight day of growth, the resin was filtered from the bacterial culture under vacuum, desalted by rinsing with Milli-Q water, and allowed to dry overnight at room temperature. Secreted metabolites were eluted from the resin by two extractions in 100 mL of (1:1) MeOH/CH₂Cl₂, followed by 100 mL of MeOH. Both extractions were combined and dried under vacuum centrifugation (ThermoSavant). The dried extract was resuspended in DMSO at 100 mg/mL and stored at -85 °C until further testing in bacterial susceptibility assays against *E. coli* strains overexpressing RND transporters.[15]

Once isolate B158 was determined to contain active compound(s), a total of 118.5 L of B158 culture was grown in 1.5 L Fernbach flasks to obtain enough crude material for structural elucidation. Briefly, 2 mL of “starter” culture in TSW was used to inoculate Fernbach flasks containing 1.5 L of 1 x TSY and allowed to grow for 7 days at 100 rpm at 23 °C. On day 7, 20 mL (approximately weight = 7.8 g) of 1:1 mixture of sterile Amberlite XAD-7 and XAD-16 resin that had been extensively washed in organic solvent, dried, and autoclaved was added to each 1.5 L culture. After 24 h, the resin was

filtered from the bacterial culture through combusted stainless steel mesh under gentle vacuum (<5 in Hg), desalted by rinsing with MilliQ water, pooled, and dried overnight at room temperature. Each 1.5 L resin equivalent was extracted with 100 mL of (1:1) MeOH/CH₂Cl₂, followed by MeOH. This crude extract was dried under vacuum centrifugation and stored at -85 °C until bioassay guided fractionation.

4.5 Bioassay Guided Fractionation of Exudates from B158

Assessment of crude extracts via MDR reversal assays with *E. coli* MDR strains MG1655 Δ BC/pABM, and MG1655 Δ BC/pXYM separately overexpressing two Resistance Nodulation cell Division (RND) transporters indicated the presence of active compound(s) and was completed following previously published methods.[15] Crude extract was applied to a silica gel column (40-60 μ m, 60Å) and eluted with a step-gradient of isooctane, (4:1) isooctane/ EtOAc, (3:2) isooctane/EtOAc, (2:3) isooctane/EtOAc, (1:4) isooctane/EtOAc, EtOAc, (1:1) EtOAc/MeOH, and 100% MeOH, yielding eight fractions. Bacterial susceptibility testing in the INT assay indicated activity to be localized to the following three gradients: (1:4) isooctane/EtOAc, EtOAc, (1:1) EtOAc/MeOH. Material from these three fractions were combined and the material was further separated by solid-phase extraction (Supelclean ENVI-18) eluting with a step-gradient of (20:1) water/MeCN, (4:1) water/MeCN, (3:2) water/MeCN, (2:3) water/MeCN, (1:20) water/MeCN, and acetone. All solvents, except acetone, were acidified with 0.1% formic acid (FA). The active fraction eluted with (4:1) and (3:2) water/MeCN gradients. The active fraction totaling (700 mg) was separated further into 96-deep well plates by semipreparative HPLC using an Agilent 1200 series HPLC and a Phenomenex Luna® 5 μ m C₁₈ (2) 100 Å, LC column (250 x 10 mm) as the stationary

phase, heated to 30 °C and a gradient of water and MeCN with a flow rate of 4 mL/min. All solvents were acidified with 0.1% FA. Contents of the 96-deep well plate were concentrated *in vacuo* and tested in the INT assay. Two fractions were found to contain the desired activity, wells G6 to H6 (most active) totaling 32.4 mg and H8, A9, B9 (minor activity) totaling 25.1 mg, which eluted (3:2) water/MeCN and (1:1) water/MeCN, respectively. The fraction containing wells G6 to H6 was further separated by analytical HPLC using an Agilent 1260 series LC-MS and a Phenomenex Kinetex 2.6 μm C18 100Å, LC column (150 x 2.1 mm) as the stationary phase, heated to 30 °C and a gradient of water and MeOH with a flow rate of 0.2 mL/min. All solvents were acidified with 0.1% FA. Compound **1** eluted at 13.2 min corresponding to (7:3) water/MeOH and yielded a total of 1 mg of white powder.

4.6 Compound Data

3,6-dihydroxy-1,2-benzisoxazole (1): Synthesis of 3,6-dihydroxy-1,2-benzisoxazole was performed as previously reported.[35] Hydroxylamine hydrochloride (2.50 g, 36.0 mmol) was added to a stirring solution of KOH (2.69 g, 47.9 mmol) in water (30 mL) at room temperature. Methyl 2,4-dihydroxy-benzoate (1.01 g, 6.0 mmol) dissolved in 1,4 dioxane (5 mL) was added dropwise to the hydroxylamine solution and stirred for 24 h at room temperature. After removing the 1,4 dioxane *in vacuo*, the pH was adjusted to 1 using 3N HCl and extracted with EtOAc (3 x 50 mL). The organic layers were combined and dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The resulting residue was purified by silica gel flash chromatography using a gradient of 20-100% EtOAc/hexanes to afford 2,4-dihydroxy-benzohydroxamic acid (765.7 mg, 76% yield).

2,4-dihydroxy-benzohydroxamic acid (161.4 mg, 0.96 mmol) was dissolved in anhydrous tetrahydrofuran (10 mL) under N₂. Dry 1,1'-carbonyldiimidazole (CDI) (489.2 mg, 3.0 mmol) was added to the mixture and heated at reflux conditions for 30 min. Et₃N (210 μ L, 1.5 mmol) was then added and the reaction proceeded at reflux for 18 h. The THF was removed *in vacuo* and the resulting residue was reconstituted in 1N HCl and extracted with EtOAc (3 x 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated. The resulting residue was purified by silica gel flash chromatography using a gradient of 30-50% EtOAc with 0.1% FA/hexanes to afford 3,6-dihydroxy-1,2-benzisoxazole (107.7 mg, 74% yield, overall yield 56%). Prior to bioassay, this product was further purified by HPLC with an isocratic method of 20% MeOH in H₂O with 0.1% FA to afford pure **1** (white powder). ¹H-NMR (500 MHz, CD₃OD), ¹³C-NMR (125 MHz, CD₃OD) see **Table 1**. HRMS calcd for C₇H₄NO₃: 150.0191. Found [M-H] 150.0206.

3-hydroxy-1,2-benzisoxazole (2): Compound **2** was prepared from commercially available methyl salicylate (methyl 2-hydroxy-benzoate) according to the scheme used to prepare **1**. **2** was further purified by semi-preparative HPLC using an isocratic method of 50% MeOH in H₂O with 0.1% FA to afford pure **2** (white powder). ¹H-NMR (500 MHz, (CD₃)₂SO) δ 7.31 (1H, t, *J* = 7.0), 7.54 (1H, d, *J* = 8.2), 7.59 (1H, t, *J* = 7.9), 7.75 (1H, d, *J* = 8.0), 12.45 (1H, br s). ¹³C-NMR (125 MHz, (CD₃)₂SO) δ 110.0, 114.6, 121.4, 122.9, 130.5, 163.0, 165.4. HRMS calcd for C₇H₄NO₂: 134.0242. Found [M-H] 134.0251.

3-hydroxy-6-fluoro-1,2-benzisoxazole (3): Compound **3** was prepared from commercially available methyl 2-hydroxy-4-fluoro-benzoate according to the scheme used to prepare **1**. **3** was further purified by semi-preparative HPLC using the method: 12

min gradient from 55-100% MeOH in H₂O with 0.1% FA to yield pure **3** (white powder). ¹H-NMR (500 MHz, CD₃OD) δ 7.09 (1H, td, J = 1.5, 9.1), 7.22 (1H, dd, J = 1.5, 9.1), 7.70 (1H, dd, J = 5.2, 8.9). ¹³C-NMR (125 MHz, CD₃OD) δ 98.3 (d, J = 27.7), 112.8, 113.0 (d, J = 25.7), 123.9 (d, J = 11.5), 165.7 (d, J = 14.4), 166.3 (d, J = 249.0), 167.0. HRMS calcd for C₇H₃FNO₂: 152.0148. Found [M-H]: 152.0271.

3-hydroxy-6-(benzyloxy)-1,2-benzisoxazole (4): Commercially available methyl 2,4-dihydroxy-benzoate (1.0 g, 5.92 mmol) was dissolved in 10 mL acetone and treated with K₂CO₃ (1.0 g, 7.24 mmol) and NaI (0.25 g, 1.67 mmol). The resulting suspension was stirred for 10 min under N₂ and then benzyl bromide (700 μ L, 5.85 mmol) was added dropwise. The reaction mixture was stirred for 24 h at ambient temperature. After removing the acetone *in vacuo*, the resulting residue was resuspended in H₂O and extracted with EtOAc (4 x 40 mL). The organic layers were combined, dried over Na₂SO₄, and concentrated *in vacuo*. The residue (1.8 g) was purified by silica gel flash chromatography using a gradient of 10-100% EtOAc in hexanes to afford methyl 2-hydroxy-4-benzyloxy-benzoate (687.4 mg, 45.5% yield) as white crystals. Methyl 2-hydroxy-4-benzyloxy-benzoate was then further elaborated per **Scheme 1** to afford analog **4**, which was further purified by semi-preparative HPLC using an isocratic method of 50% MeOH in H₂O with 0.1% FA (white powder). ¹H-NMR (500 MHz, (CD₃)₂SO) δ 5.17 (2H, s), 6.94 (1H, d, J = 8.7), 7.15 (1H, s), 7.34 (1H, t, J = 7.4), 7.40 (2H, t, J = 7.4), 7.46 (2H, d, J = 7.4), 7.60 (1H, d, J = 8.6). ¹³C-NMR (125 MHz, (CD₃)₂SO) δ 69.9, 94.5, 108.3, 113.4, 122.0, 127.9 (2C), 128.1, 128.5 (2C), 136.5, 161.1, 164.9, 165.7. HRMS calcd for C₁₄H₁₀NO₃: 240.0661. Found [M-H] 240.0564.

3-hydroxy-6-amino-1,2-benzisoxazole (5): Compound **5** was prepared from commercially available methyl 2-hydroxy-4-amino-benzoate (AK Scientific, Union City, CA) according to the scheme used to prepare **1** with minor modification. The ring closing step was accomplished with CDI/MeCN instead of CDI/THF/Et₃N. The resulting mixture was purified using semi-preparative HPLC with a gradient method from 10-55% MeOH in H₂O with 0.1% FA (white powder). ¹H-NMR (500 MHz, (CD₃)₂SO) δ 5.80 (2H, s), 6.39 (1H, d, J = 1.4), 6.50, (1H, dd, J = 1.5, 8.4), 7.30 (1H, d, J = 8.4). ¹³C-NMR (125 MHz, (CD₃)₂SO) δ 91.2, 103.6, 111.8, 121.8, 152.3, 165.6, 165.9. HRMS calcd for C₇H₅N₂O₂: 149.0351. Found [M-H] 149.0323.

3-hydroxy-6-methoxy-1,2-benzisoxazole (6): Compound **6** was purchased commercially (Sigma Aldrich, St. Louis, MO).

3,4,6-trihydroxy-1,2-benzisoxazole (7): Compound **7** was prepared from commercially available methyl 2,4,6-trihydroxy-benzoate (abcr GmbH, Karlsruhe, Germany) according to the scheme used to prepare **1**. **7** was further purified from a mixture by semi-preparative HPLC with a gradient method of 25-100% MeOH in H₂O with 0.1% FA (white powder). ¹H-NMR (500 MHz, (CD₃)₂SO) δ 6.08 (1H, d, J = 1.3), 6.14 (1H, d, J = 1.3), 10.22 (3H, br s). ¹³C-NMR (125 MHz, (CD₃)₂SO) δ 86.8, 96.6, 97.3, 153.8, 161.9, 165.2, 166.6. HRMS calcd for C₇H₄NO₄: 166.0140. Found [M-H] 166.0151.

N-methyl-6-hydroxy-1,2-benzisoxazol-3(2H)-one (8) and N-methyl-6-methoxy-1,2-benzisoxazol-3(2H)-one (9): Compounds **8** and **9** were prepared from **1** in a one-step synthesis and purification. **1** (46 mg, 0.33 mmol) was dissolved in dry MeCN (4 mL) and K₂CO₃ (91 mg, 0.66 mmol) was suspended in the solution. Suspension was stirred rapidly under N₂ and excess CH₃I (260 μ L, 4.2 mmol) was added dropwise and stirred for 18 h at

room temperature. The reaction was quenched by removing MeCN *in vacuo* and the resulting residue was dissolved in 1N HCl. This solution was extracted with EtOAc (3 x 30 mL) and the combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo*. The resulting products were purified by semi-preparative HPLC using a 12 min gradient from 35-80% MeOH in H₂O with 0.1% FA to afford **8** (12.1 mg, white powder) and **9** (8.5 mg, white powder).

Compound **8**: ¹H-NMR (500 MHz, (CD₃)₂SO) δ 3.46 (3H, s), 6.68 (1H, d, *J* = 1.6), 6.57 (1H, dd, *J* = 1.6, 8.4), 7.55 (1H, d, *J* = 8.4), 10.73 (1H, br s). ¹³C-NMR (125 MHz, (CD₃)₂SO) δ 33.3, 95.3, 106.8, 113.4, 124.7, 162.3, 163.4, 164.2. HRMS calcd for C₈H₈NO₃: 166.0504. Found [M+H]: 166.0216.

Compound **9**: ¹H-NMR (500 MHz, (CD₃)₂SO) δ 3.51 (3H, s), 3.86 (3H, s), 6.89 (1H, dd, *J* = 2.1, 8.6), 7.05 (1H, d, *J* = 2.0), 7.64 (1H, d, *J* = 8.6). ¹³C-NMR (125 MHz, (CD₃)₂SO) δ 33.2, 56.1, 93.8, 108.1, 113.2, 124.5, 162.2, 163.7, 164.5. HRMS calcd for C₉H₁₀NO₃: 180.0661. Found [M+H]: 180.0341.

6-hydroxy-2-benzoxazolinone (10): Compound **10** was purchased commercially (abcr GmbH, Karlsruhe, Germany).

Supplementary Materials: The following are available online at.... Purification and dereplication details of compounds **11** and **12** and pertinent NMR and MS data are included.

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CHAPTER 4

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Uropathogenic *Escherichia coli* Metabolite-Dependent Quiescence and Persistence May
Explain Antibiotic Tolerance During Urinary Tract Infection

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Running Title: *E. coli* CFT073 Quiescence and Persistence In Vitro

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Abstract

In the present study it is shown that although *E. coli* CFT073, a human uropathogenic (UPEC) strain, grows in liquid glucose M9 minimal medium, it fails to grow on glucose M9 minimal medium agar plates seeded with $\leq 10^6$ CFU. The cells on glucose plates appear to be in a “quiescent” state that can be prevented by various combinations of lysine, methionine, and tyrosine. Moreover the quiescent state is characteristic of ~ 80% of *E. coli* phylogenetic group B2 multilocus sequence type 73 strains as well as 22.5% of randomly selected UPEC strains isolated from community acquired urinary tract infections in Denmark. In addition, *E. coli* CFT073 quiescence is not limited to glucose, but occurs on agar plates containing a number of other sugars and acetate as sole carbon sources. It is also shown that a number of *E. coli* CFT073 mini-Tn5 metabolic mutants (*gnd*, *gdhA*, *pykF*, *sdhA*, and *zwf*) are non-quiescent on glucose M9 minimal agar plates and that quiescence requires a complete oxidative TCA cycle. In addition, evidence is presented that although *E. coli* CFT073 quiescence and persistence in the presence of ampicillin are alike in that both require a complete oxidative TCA cycle and that each can be prevented by amino acids, *E. coli* CFT073 quiescence occurs in the presence or absence of a functional *rpoS* gene, whereas maximal persistence requires a non-functional *rpoS*. Our results suggest that interventions targeting specific central metabolic pathways may mitigate UPEC infections by interfering with quiescence and persistence.

Importance

Recurrent urinary tract infections (UTIs) affect 10 to 40% of women. In up to 77% of those cases the recurrent infections are caused by the same uropathogenic *E. coli* (UPEC) strain that caused the initial infection. Upon infection of urothelial transitional cells in the bladder, UPEC appear to enter a non-growing quiescent intracellular state that is thought to serve as a reservoir responsible for recurrent UTIs. Here we report that many UPEC strains enter a quiescent state when $\leq 10^6$ CFU are seeded on glucose M9 minimal medium agar plates and show that mutations in several genes involved in central carbon metabolism prevent quiescence as well as persistence, possibly identifying metabolic pathways involved in UPEC quiescence and persistence in vivo.

Introduction

Uncomplicated urinary tract infections (UTIs) affect about 25% of women in their lifetime and at least 80% of those infections are caused by uropathogenic *E. coli* (UPEC) (1). Recurrent UTIs affect between 10% and 40% of women (2) and in up to 77% of those cases the recurrent infections are caused by the same UPEC strain that caused the initial infection (3, 4). UPEC infections generate annual costs in excess of two billion dollars in the United States alone, placing a significant burden on the health care system (5). Although the causes of recurrent UTI are complex (6), it appears that UPEC can bind to, enter, and replicate within superficial facet cells in the human and mouse bladder epithelium, resulting in biofilm-like intracellular communities (IBCs) (6, 7). IBCs escape from infected superficial facet cells within hours of development (6). The superficial facet cells then exfoliate, exposing underlying transitional epithelial cells, which can be

infected with IBC-derived UPEC progeny (6, 8). Upon infection of urothelial transitional cells, UPEC appear to enter a non-growing quiescent intracellular state (6, 8). These quiescent UPEC cells have been called QIRs (**Q**uiescent **I**ntracellular **R**eservoirs) (6) and it is thought that QIRs are a major cause of recurrent UTIs (6, 8). QIRs also help to explain why antibiotics have failed to eradicate UPEC reservoirs in the bladders of mice, since quiescent UPEC may not be readily affected by antibiotics (6, 8).

The quiescence of QIRs and their insensitivity to antibiotics is reminiscent of the persister state (8, 9, 10, 11). Persister cells are dormant cells formed in normal microbial populations as small subpopulations that are highly tolerant to antibiotics, but upon regrowth in the absence of antibiotics regain full sensitivity (11). Persisters appear to play a major role in the ability of chronic infections to withstand antibiotic treatment (11). In the present study, we report that when $\leq 10^6$ CFU of *E. coli* CFT073, the prototypic UPEC strain, as well as ~ 80% of phylogenetic group B2 multilocus sequence type 73 (ST73) strains, of which *E. coli* CFT073 is a member, are plated on M9 minimal agar plates containing glucose as sole carbon and energy source, they appear to enter a “quiescent” state and that mutations in specific metabolic genes appear to prevent that state. In addition, we show that *E. coli* CFT073 quiescence also occurs in the presence of a number of other sugars and acetate as sole carbon sources and that a complete TCA cycle is required for both generation of *E. coli* CFT073 quiescent cells on glucose plates and for formation of persister cells generated in liquid glucose minimal medium in the presence of ampicillin.

Materials and Methods

Bacterial strains. The *E. coli* CFT073, MG1655, and Nissle 1917 strains used in this study are listed in Table 1. The original *E. coli* K-12 strain was obtained from a stool sample from a convalescing diphtheria patient in Palo Alto, CA, in 1922 (12). The sequenced *E. coli* MG1655 strain (CGSC 7740) was derived from the original K-12 strain, having only been cured of the temperate bacteriophage lambda and the F plasmid by means of UV light and acridine orange treatment (12). *E. coli* Nissle 1917 was originally isolated during World War I from a soldier who escaped a severe outbreak of diarrhea (13). It has a beneficial effect on several types of intestinal disorders, is well tolerated by humans, and has been marketed as a probiotic remedy against intestinal disorders in several European countries since the 1920s (13). *E. coli* strains tested for quiescence in the Testing additional *E. coli* strains for quiescence on glucose plates and the Testing additional ST73 strains for quiescence on glucose plates sections in Results are from the E. V. Sokurenko collection and the *E. coli* strains used in the Testing 40 UPEC strains isolated from community acquired UTIs in Denmark for quiescence on glucose plates section in Results are from the N. Frimodt-Møller/ K.L. Nielsen collection.

Media. LB broth (Lennox) (Difco Laboratories) and LB agar (Lennox) (Difco Laboratories) were used for routine cultivation. SOC medium was prepared as described by Datsenko and Wanner (14). Liquid M9 minimal medium (15) and M9 minimal medium agar plates were supplemented with reagent grade (Sigma-Aldrich, Inc.) L-arabinose (0.2%, w/v), *N*-acetyl-D-glucosamine (0.2%, w/v), L-fucose (0.2%, w/v), D-fructose (0.2%, w/v), D-glucose (0.2%, w/v), D-galactose (0.2%, w/v), D-gluconate

(0.2%, w/v), glycerol (0.2%, v/v), D-mannose (0.2%, w/v), maltose (0.2%, w/v), D-ribose (0.2%, w/v), D-xylose (0.2%, w/v), potassium acetate (0.4%, w/v), sodium succinate (0.4%, w/v), and various amino acids, as indicated. Since Difco Bacto agar contains impurities, M9 minimal medium agar plates were made with 1.6% Difco Agar Noble (Difco).

Lawn assay for quiescence. All *E. coli* strains were streaked from -80°C stored LB broth grown cultures diluted 1:1 with 50% (v/v) glycerol to LB agar plates which were incubated overnight at 37°C. A loopful of cells from each streak plate was then grown overnight in 10 ml of 0.4% glucose M9 minimal medium at 37°C with shaking in 125 ml tissue culture bottles. Routinely, 10⁵ CFU from an overnight culture was added to a tube containing 3 ml of liquid overlay medium (0.2% glucose M9 minimal medium, 0.7% Difco Agar Noble at 45°C). Each tube containing inoculated overlay medium was immediately poured on a pre-warmed (37°C) 0.2% glucose M9 minimal medium agar plate. Inoculated overlays were allowed to solidify (with lids slightly ajar) for 1 h at room temperature. In addition, as indicated, solidified inoculated overlays were stabbed with colonies of *E. coli* MG1655 grown on 0.4% glucose M9 minimal medium agar plates using sterile toothpicks or were spotted with 5 µl to 20 µl of filtered *E. coli* MG1655 culture supernatant, human urine, or defined amino acid solutions. Spots were allowed to dry prior to incubation of plates. Plates were incubated for 24 h or 48 h at 37°C, as indicated. Strains that grew as lawns on 0.2% glucose M9 minimal medium agar plates were considered to be non-quiescent and strains that grew in liquid 0.2% glucose M9 minimal medium, failed to grow on 0.2% glucose M9 minimal medium agar

plates, but were stimulated to grow around *E. coli* MG1655 stabs were considered to be quiescent (see Results).

Insertional mutagenesis. Mini-Tn5 Km (kanamycin) mutants were constructed by insertional mutagenesis as described previously (16). Briefly, the donor *E. coli* ATM161 (17) carrying the suicide vector pUT, which contains the mini-Tn5 Km transposon, was conjugated with the recipient *E. coli* CFT073 Str^R (Table 1) in the following manner. The donor and recipient strains were grown overnight, with shaking, in LB broth at 30°C. Aliquots of 100 µl of each culture were mixed together in 5 ml of 10 mM MgSO₄ and filtered through a 0.45 µm pore size membrane filter (EMD Millipore). The filter was placed on the surface of an LB agar plate and incubated for 5 h at 37°C. Following incubation, the bacteria on the filter were suspended in 5 ml of 10 mM MgSO₄, and 100 µl aliquots of the suspension were plated on LB agar containing streptomycin sulfate (100 µg/ml) and kanamycin sulfate (80 µg/ml) and plates were incubated for 18 h at 37°C. Individual mini-Tn5 Km mutant colonies were toothpicked to 2 LB agar plates, one lacking and one containing ampicillin sodium salt (100 µg/ml, Sigma-Aldrich, Inc.). Colonies that were ampicillin sensitive, signifying loss of the pUT suicide plasmid, were toothpicked to sterile 16 mm diameter culture tubes containing 250 µl of 0.2% glucose M9 minimal medium. Culture tubes were incubated overnight at 37°C with shaking, 5 ml of M9 minimal medium lacking a carbon source was then added to each tube, and 3 µl from each tube was spotted on a 0.2% glucose M9 minimal medium agar plate. Spots were allowed to dry (with lids slightly ajar) and plates were incubated overnight at 37°C. Each spotted mini-Tn5 Km mutant that grew was retested by seeding 10⁵ CFU of an overnight 10 ml liquid 0.4% glucose M9 minimal medium culture on a 0.2% glucose M9

minimal medium agar plate and incubating at 37°C for 24 hours. The gene inactivated in each of the mini-Tn5 Km mutants that grew as a lawn was determined by arbitrary PCR (18), as described below. In addition, to be sure that the mini-Tn5 Km insertion was the cause of the ability of the individual mutants to grow on glucose plates, the insertion in each mutant was transferred into a fresh *E. coli* CFT073 Str^R background by the method of Wanner and Datsenko (14). Each mutant thus obtained was confirmed for the ability to grow as a lawn on glucose plates and for the position of the insertion within the *E. coli* CFT073 chromosome by both PCR and sequencing (see Table 2 for primers). Five confirmed mutants were isolated from approximately 2000 mini-Tn5 Km mutants tested.

Arbitrary PCR. Arbitrary PCR was performed as described previously (18). Genomic DNA was isolated from *E. coli* CFT073 mini-Tn5 Km mutants using the Wizard® Genomic DNA Purification Kit (Promega). The first round of PCR was performed in 25 µl reactions (1X Standard *Taq* Reaction Buffer (New England Biolabs, Inc.), 2 mM dNTPs, 100 µM arbitrary primer 1 (5'-GGCCACGCGTCGACTAGTACNNNNNNNNNNNGATAT-3'), 10 µM Tn5-specific primer (5'-TCTGGATTTTCGATCACGGCAC G T-3'), *Taq* DNA Polymerase (2 units, New England Biolabs, Inc.) and DNA from 1.25 µl of an overnight LB broth culture. The first round cycling conditions were: (i) 4 min at 95°C; (ii) 6 x (30s at 95°C, 30s at 30°C, 1.5 min at 72°C 1.5); (iii) 30 x (30s at 95°C, 30s at 45°C, 2 min at 72°C); (iv) 4 min at 72°C. The second round of PCR used standard conditions and cycling as follows: (i) 4 min at 95°C; 35 x (30s at 95°C, 30s at 55°C, 2 min at 72°C), (iii) 4 min at 72°C with 0.5 µl of the first round PCR product as template and 10 µM arbitrary primer 2 (5'-GGCCACGCGTCG ACTAGTAC-3') and 10 µM of a second Tn5-specific primer (5'-

TTACCGAGAGCTTGGTAC CCAGTC-3'). The second round of PCR reactions were column purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced using a third Tn5-specific primer (5'-GTACCCAGTCTGTGTGAGCAGG-3'). DNA sequencing was done at the URI Genomics and Sequencing Center, University of Rhode Island, Kingston, RI using an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). A BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems) was used for the sequencing reactions. Sequences were compared with the GenBank DNA sequence database using the BLASTX program.

Identification and quantitation of amino acids in *E. coli* MG1655 and *E. coli*

CFT073 50-fold concentrated culture supernatants. *E. coli* MG1655 was grown overnight in five 10 ml cultures in liquid 0.4% glucose M9 minimal medium with shaking at 37°C in 125 ml tissue culture bottles. The cultures were pooled and cells were centrifuged for 10 min at 8000 x g and resuspended in 1 ml of 0.2% glucose M9 minimal medium. The 50-fold concentrated cultures were incubated standing overnight at 37°C in 1.5 ml centrifuge tubes (CELLTREAT Scientific Products). Cells were then centrifuged at 16,000 x g for 3 min and the supernatant was removed and filtered free of bacteria using a 0.22 µm MCE syringe filter (Fisherbrand). *E. coli* CFT073 concentrated culture supernatants were prepared identically.

Amino acids in 50-fold concentrated culture supernatants were identified and quantified using a slightly modified version of a method described by Yuan et al. (19). The method uses the AccQ Tag Amino Acid analysis method (Waters Corp., Milford, MA, USA) with a pre-column derivatization kit (<http://www.waters.com/webassets/cms/support/docs/wat0052881.pdf>). Acid hydrolysis

was not included in the derivatization step to assure that only free amino acids were quantified. The method uses HPLC to separate the derivatized amino acids and a fluorescence detector to identify and quantify them. The chromatographic method using AccQ Tag Eluent A (solvent A) and 60% acetonitrile in water (solvent B) is as follows: consecutive linear gradients of 0-2% B over 0.5 min; 2-7% B over 14.5 min; 7-10% B over 4 min; 10-20% B over 11 min; 20-36% B over 10 min; and 36-100% B over 5 min. The method totals 45 min of gradients and an additional 11 min washout phase. The flow rate was constant at 0.7 ml/min using a Waters AccQ Tag column (150 x 3.9 mm) held at a 40°C and injecting 5 µl aliquots. The fluorescence detector was set to an excitation wavelength of 250 nm and emission wavelength of 395 nm per manufacturer's instructions. Amino acids were identified by comparison to derivatized amino acid reference standards in the following order: tryptophan, 6-aminoquinoline (AMQ, a product of the derivatization process), aspartic acid, serine, glutamic acid, glycine, histidine, ammonium ion, arginine, threonine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine, phenylalanine. A calibration curve was generated to quantify each amino acid present.

Persister assay. Overnight cultures in liquid 0.4% glucose M9 minimal medium were grown as described in the Lawn assay for quiescence section, above. Cultures were then diluted 20-fold into 10 ml of fresh 0.2% glucose M9 minimal medium ($A_{600} = 0.1$, $\sim 10^8$ CFU/ml) in both the presence and absence of ampicillin sodium salt (100 µg/ml, Sigma-Aldrich, Inc.). The cultures were incubated with shaking at 37°C and viable counts on LB agar plates were determined at 2, 4, 6, and 24 h. To determine whether cells surviving at 24 h in the presence of ampicillin were persister cells or ampicillin resistant mutants, 5

ml were washed free of ampicillin by centrifuging at 16,000 x g for 3 min, washing twice in 5 ml of fresh 0.2% glucose M9 minimal medium, and resuspending in 5ml of LB broth. Each 5 ml culture was then grown for 2.25 h at 37°C with shaking in a 125 ml tissue culture bottle at which time a sample was taken for viable counts. Ampicillin sodium salt (100 µg/ml) was then added to each culture and 4 h later a sample was again taken for viable counts on LB agar plates. Plates were incubated at 37°C for 18 h prior to counting.

Photography. Images of agar plates were made using a BIO-RAD Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software.

Statistics. Means and standard deviations of data presented in Table 4 and in Figures 4, 5, 7, 8, and 9 were compared by a two-tailed Student t test. *P* values of ≤ 0.05 were interpreted as indicating a significant difference.

Results

***E. coli* CFT073, a UPEC strain, and *E. coli* Nissle 1917, a closely related probiotic strain, grow in liquid glucose M9 minimal medium, but fail to grow on glucose M9 minimal medium agar plates.** We were attempting to determine which colicins and microcins are active on the sequenced *E. coli* CFT073, a phylogenetic group B2 multilocus sequence type 73 (ST73) UPEC strain (20, 21), and the closely related ST73 probiotic strain, *E. coli* Nissle 1917 (22). As expected, we found that after overnight incubation at 37°C, both strains formed lawns of growth on 0.2% glucose M9 minimal medium agar plates (hereafter called glucose plates) when either 10^7 or 10^8 CFU were plated from an overnight 0.4 % glucose M9 minimal medium liquid culture.

Unexpectedly, however, when 10^6 CFU or fewer were plated on glucose plates they failed to form lawns or colonies after 24 h and 48 h of incubation at 37°C. This result was unusual in that a number of human commensal *E. coli* strains, including MG1655, HS, F-18, EFC1, and EFC2 (Table 1), and the O157:H7 strain *E. coli* EDL933 (Table 1) tested at 10^5 CFU grew as lawns on the glucose plates after 24 hours at 37°C and viable counts could be determined for each strain on glucose plates (not shown). That all the *E. coli* strains tested for growth on glucose plates are spontaneous streptomycin resistant mutants (Table 1), but only *E. coli* CFT073 and *E. coli* Nissle 1917 failed to form lawns on glucose plates seeded with $\leq 10^6$ CFU, makes it highly unlikely that point mutations in ribosomal proteins play a role in the observed lack of growth.

Toothpicking a colony of *E. coli* MG1655 grown on glucose plates onto glucose plates seeded with 10^5 or 10^6 CFU of *E. coli* CFT073 or *E. coli* Nissle 1917 resulted in *E. coli* CFT073 and *E. coli* Nissle 1917 growth around the toothpicked colonies after incubation for 24 h at 37°C, but not anywhere else on the plates (Fig. 1A) (toothpicking colonies in this manner is a normal procedure used in colicin and microcin testing). No growth was observed around toothpicked *E. coli* MG1655 colonies grown on glucose plates that had not been seeded with *E. coli* CFT073 or *E. coli* Nissle 1917 (not shown). These results suggested that toothpicked *E. coli* MG1655 was secreting a molecule(s) (hereafter called the MG1655 stimulus) as it grew on glucose plates that was either preventing *E. coli* CFT073 and *E. coli* Nissle 1917 death or was preventing them from entering a “quiescent” state. Incubating the plates that had been seeded with 10^5 CFU of *E. coli* CFT073 or *E. coli* Nissle 1917 for 48 h at 37°C resulted in a much larger region of growth around the toothpicked *E. coli* MG1655 than at 24 h (Fig. 1B), suggesting that

cells could be stimulated to grow after 24 hours of incubation, thereby favoring the quiescence hypothesis and suggesting either that the MG1655 stimulus was diffusible and active at a very low concentration or that growing *E. coli* CFT073 and *E. coli* Nissle 1917 also secreted the stimulus. Also, when glucose plates seeded with 10^5 or 10^6 CFU of *E. coli* CFT073 or *E. coli* Nissle 1917 were incubated for 24 h at 37°C prior to toothpicking *E. coli* MG1655 to those plates, *E. coli* CFT073 and *E. coli* Nissle 1917 growth was observed surrounding the toothpicked *E. coli* MG1655 colonies after an additional 24 h incubation at 37°C (not shown). Therefore, many of the *E. coli* CFT073 and *E. coli* Nissle 1917 cells were alive, but “quiescent” on glucose plates for at least 24 hours. Importantly, quiescence is not observed when Difco Bacto agar is used in glucose plates instead of Difco Agar Noble, suggesting impurities in the former allow growth. It should be noted that at the present time we do not know whether it is alive or dead *E. coli* MG1655 cells that are the source of the stimulus as the toothpicked cells grow on the glucose plates. Also, it should be mentioned that *E. coli* CFT073 and *E. coli* Nissle 1917 quiescence is not dependent on using 0.2% glucose M9 minimal medium agar plates, identical results were obtained using 0.4% glucose M9 minimal medium agar plates (not shown).

Testing additional *E. coli* strains for quiescence on glucose plates. *E. coli* can be separated into four major phylogenetic groups (A, B1, B2 and D) and two additional phylogenetic groups that have recently been defined: phylogenetic group AxB1, containing strains that derive most of their ancestry from A and B1, and phylogenetic group ABD, containing a heterogeneous set of strains with multiple sources of ancestry (23). Thirty *E. coli* strains representing various multilocus sequence types (ST) of the 6

phylogenetic groups, i.e. two ST10 and two ST453 strains from phylogenetic group A; two ST58, two ST410, and two ST101 strains from phylogenetic group B1; two ST73, two ST95, and two ST131 strains from phylogenetic group B2; two ST69, two ST354, and two ST648 strains from phylogenetic group D; two ST90 and two ST642 strains from phylogenetic group AxB1; two ST62 and two ST117 strains from phylogenetic group ABD were grown in liquid glucose M9 minimal medium and the 30 strains were tested for the ability to grow on glucose plates seeded with 10^5 CFU and to respond to the MG1655 stimulus. Of the 30 strains, 2 failed to grow on glucose plates and those strains responded to the MG1655 stimulus. The 2 strains that failed to grow on glucose plates were ST73 strains. ST73 is a very common UPEC lineage, accounting for 11% and 16.6% of UPEC isolated from patients in 2 recent studies (24, 25). Importantly, *E. coli* CFT073 and *E. coli* Nissle 1917 are also ST73 strains.

Testing additional ST73 strains for quiescence on glucose plates. Since it appeared that quiescence on glucose agar plates may be characteristic of the ST73 lineage, 40 additional ST73 strains were tested for the ability to grow on glucose plates seeded with 10^5 CFU and to respond to the MG1655 stimulus. Two of the strains failed to grow overnight in liquid glucose M9 minimal medium, but of the 38 strains that grew, 30 (78.9%) failed to grow on glucose plates, but responded to the MG1655 stimulus. Therefore, the vast majority of ST73 strains, a major UPEC lineage (24, 25), are quiescent on glucose plates.

Testing 40 UPEC strains isolated from community acquired UTIs in Denmark for quiescence on glucose plates. Forty randomly selected UPEC strains isolated from community acquired UTIs in Denmark were tested for the ability to grow on glucose

plates seeded with 10^5 CFU and to respond to the MG1655 stimulus. Of the 40 UPEC strains tested, all grew overnight in liquid glucose M9 minimal medium, but 9 failed to grow on glucose plates (22.5%) unless stimulated to do so by the MG1655 stimulus. Three of the 9 strains that failed to grow on glucose plates were ST73 strains (5 of the 40 UPEC strains tested [12.5%] were ST73) and 3 of the 9 strains that failed to grow on glucose plates were ST141 strains (3 of the 40 UPEC strains tested were ST141 strains [7.5%], a group not represented in the original 30 strains tested). The 3 remaining strains that failed to grow on glucose plates (ST104, ST394, and ST998) were not represented in the original 30 strains tested and each was represented only once among the 40 UPEC strains tested (2.5% each). It therefore appears that the inability to grow on glucose plates, yet respond to the MG1655 stimulus, is not limited to the ST73 group.

The inability of *E. coli* CFT073 to grow on minimal agar plates is not limited to glucose as sole carbon source. *E. coli* CFT073 was tested for the ability of 10^5 CFU to grow on M9 minimal agar plates containing 0.2% acetate, arabinose, fructose, fucose, galactose, gluconate, glycerol, N-acetylglucosamine, maltose, mannose, ribose, and xylose as sole carbon sources. *E. coli* CFT073 grew overnight in liquid M9 minimal medium containing each carbon source, but 10^5 CFU only grew as a lawn on agar plates containing glycerol, ribose, and xylose as sole carbon sources (Fig. 2). On those plates that *E. coli* CFT073 failed to grow, it responded to the MG1655 stimulus.

Human urine, a cocktail mimicking the amino acid composition of human urine, and a cocktail mimicking amino acids present in a concentrated *E. coli* MG1655 culture supernatant prevent *E. coli* CFT073 quiescence on glucose plates. We normally toothpick a colony of *E. coli* MG1655 to a glucose plate as a source of the

MG1655 stimulus, suggesting that the stimulus is secreted on the plate as *E. coli* MG1655 grows. However, no stimulus activity was found when 5 μ l or 20 μ l of a cell-free supernatant derived from an overnight liquid glucose M9 minimal medium culture of *E. coli* MG1655 was placed on a glucose agar plate seeded with 10^5 CFU of *E. coli* CFT073 or *E. coli* Nissle 1917. Stimulus activity was observed when 5 μ l of a cell-free supernatant derived from a 50-fold concentrated *E. coli* MG1655 culture that had been incubated overnight at 37°C (see Materials and Methods) was placed on a glucose agar plate seeded with 10^5 CFU of *E. coli* CFT073 (Fig. 3A). Analysis of one such *E. coli* MG1655 cell-free supernatant revealed the presence of a number of unknown small molecules and 14 amino acids (Table 3). Importantly, 5 μ l of an amino acid cocktail identical in composition to the amino acids in the 50-fold concentrated *E. coli* MG1655 supernatant displayed similar stimulus activity to 5 μ l of the 50-fold concentrated supernatant (Fig. 3B). A cell-free supernatant derived from a 50-fold concentrated *E. coli* CFT073 culture was nearly identical to the *E. coli* MG1655 supernatant in amino acid composition, but in addition contained aspartic acid (Table 3). As expected, 5 μ l of the *E. coli* CFT073 cell-free supernatant also displayed stimulus activity on glucose plates seeded with 10^5 CFU of *E. coli* CFT073. Perhaps even more importantly, 5 μ l of sterile filtered human urine collected from one of us and 5 μ l of a cocktail mimicking the amino acid composition of human urine ([26] and Table 3) both displayed stimulus activity on glucose plates seeded with 10^5 CFU of *E. coli* CFT073 (Fig. 3C and 3D).

Lysine, methionine, and tyrosine involvement in preventing quiescence. 1.0 mM solutions of each of the 20 standard L-amino acids were prepared and 5 μ l of each was tested on glucose plates seeded with 10^5 CFU of *E. coli* CFT073. None displayed

stimulus activity. However, testing 5 μ l of single amino acid deletions from the amino acid cocktail mimicking the concentrations of amino acids in urine (Table 3) and in the 50-fold concentrated *E. coli* MG1655 supernatant (Table 3) revealed that cocktails missing lysine, methionine, and tyrosine failed to stimulate. Furthermore, although 5 μ l of 1.0 mM lysine alone, 1.0 mM methionine alone, and 1.0 mM tyrosine alone failed to stimulate (not shown), 5 μ l of mixtures of 1.0 mM each of lysine, methionine, and tyrosine (Fig. 3E) were about as stimulatory for growth of *E. coli* CFT073 on glucose plates as either the *E. coli* MG1655 amino acid cocktail (Fig. 3B) or the amino acid cocktail mimicking human urine (Fig. 3D). 5 μ l of mixtures of 1.0 mM each of D-lysine, D-methionine, and D-tyrosine failed to stimulate (not shown), demonstrating the importance of the L- forms of the 3 amino acids in preventing quiescence. Mixtures of 1.0 mM each of lysine and methionine (Fig. 3F) and 1.0 mM each of lysine and tyrosine (Fig. 3G) also stimulated *E. coli* CFT073 growth on glucose plates, although stimulation of *E. coli* CFT073 growth by the mixture of lysine and tyrosine was minimal (Fig. 3G). A mixture of 1.0 mM each of methionine and tyrosine failed to stimulate *E. coli* CFT073 growth (Fig. 3H). In summary, *E. coli* MG1655 is not required to provide the stimulus that prevents *E. coli* CFT073 quiescence, a mixture of lysine, methionine, and tyrosine, found in concentrated 50-fold concentrated *E. coli* MG1655 supernatants, is just as effective.

***E. coli* CFT073 and *E. coli* Nissle 1917, but not *E. coli* MG1655 generate high levels of persister cells in liquid glucose M9 minimal medium.** Persister cells are dormant cells formed in normal microbial populations as small subpopulations (10^{-3} to 10^{-4} %) that are highly tolerant to antibiotics, but upon regrowth in the absence of antibiotics regain

full sensitivity (11). Persister cells appear to play a role in the ability of bacteria causing chronic infections to withstand antibiotic treatment (11). Because *E. coli* CFT073 becomes quiescent on glucose plates and quiescence is reminiscent of persistence, we wondered whether *E. coli* CFT073 generates a high level of persister cells in liquid glucose M9 minimal medium. 0.4% Glucose M9 minimal medium grown overnight cultures of *E. coli* CFT073 were diluted 20-fold into fresh 0.2% glucose M9 minimal medium ($A_{600}=0.1$, $\sim 10^8$ CFU/ml) containing or lacking ampicillin (100 μ g/ml) and viable counts were followed for 24 hours at 37°C (Fig. 4). During the first 4 h of incubation, viable counts in the *E. coli* CFT073 cultures decreased 10-fold in both the presence and absence of ampicillin, i.e. from 10^8 CFU/ml to 10^7 CFU/ml. No further death occurred between 4 h and 6 h in the absence of ampicillin, and by 24 h *E. coli* CFT073 had grown to almost 10^9 CFU/ml (Fig. 4). In contrast, between 4 h and 6 h in the presence of ampicillin, viable counts decreased almost an additional 10-fold to about 10^6 CFU/ml in the *E. coli* CFT073 cultures (Fig. 4). However, there was little further *E. coli* CFT073 death in the presence of ampicillin between 6 h and 24 h (Fig. 4), suggesting the possibility that the survivors ($\sim 0.7\%$) at 24 h might be persisters. Therefore, at 24 h, *E. coli* CFT073 cultures containing ampicillin were centrifuged, washed free of the antibiotic, resuspended in LB broth, and grown at 37°C for 2.25 h to 10^8 CFU/ml, at which time ampicillin was added (100 μ g/ml). Four hours later, viable counts in the *E. coli* CFT073 LB broth cultures had dropped to 10^4 CFU/ml, suggesting that the vast majority of cells that survived ampicillin treatment in liquid glucose M9 minimal medium were indeed persister cells, still fully sensitive to ampicillin. Much like *E. coli* CFT073, *E. coli* Nissle 1917 generated a high level of persister cells in liquid glucose minimal

medium, i.e. about 2.6% at 24 h (Table 4) and the cells were still sensitive to ampicillin as described above.

Unlike *E. coli* CFT073 and *E. coli* Nissle 1917, when *E. coli* MG1655 overnight cultures were diluted 20-fold into fresh 0.2% glucose M9 minimal medium, viable counts increased immediately in the absence of ampicillin and in the presence of ampicillin decreased continuously for 6 h to a level of about 10^3 CFU/ml (10^{-3} %) and remained at that level at 24 h (Fig. 4). The *E. coli* MG1655 survivors in cultures containing ampicillin were also persister cells, i.e. when regrown in LB broth without ampicillin, they regained sensitivity. Therefore, when grown in liquid glucose M9 minimal medium, *E. coli* CFT073 and *E. coli* Nissle 1917 cultures generated about 1000-fold more persister cells than *E. coli* MG1655 cultures.

***E. coli* CFT073 generates a low level of persister cells in liquid glucose M9 minimal medium containing amino acids.** Since amino acids reversed *E. coli* CFT073 quiescence on glucose plates, we were interested in determining whether addition of a mixture of the 20 standard L-amino acids (100 µg/ml each) to cultures of *E. coli* CFT073 grown in glucose M9 minimal medium in the absence of amino acids would generate fewer persister cells. As shown in Fig. 5, in the presence of the amino acid mixture and in the absence of ampicillin, *E. coli* CFT073 viable counts increased immediately, reaching stationary phase within 4 h. Importantly, in the presence of both the amino acid mixture and ampicillin, *E. coli* CFT073 viable counts decreased continuously for 24 h to a level of about 5×10^3 CFU/ml (Fig. 5). That the survivors at 24 were persister cells was shown by the fact that when regrown in LB broth without ampicillin, they regained sensitivity, as described above. In the absence of amino acids and in the presence of

ampicillin, *E. coli* CFT073 persister cells were again generated at a level of about 10^6 CFU/ml (Fig. 5). Therefore, in the presence of amino acids, about 100-fold fewer *E. coli* CFT073 persister cells were generated than in their absence (Fig. 5).

Isolation and characterization of *E. coli* CFT073 mini-Tn5 mutants that grow on glucose plates. Since *E. coli* CFT073 grows overnight in liquid glucose M9 minimal medium, but not on glucose plates, we thought it possible that expression of one or more genes on glucose plates, but not in liquid glucose cultures might be responsible. If so, knockout of the responsible gene(s) would result in growth on glucose plates. Therefore, *E. coli* CFT073 mini-Tn5 Km (kanamycin) mutants were generated by random insertional mutagenesis (see Materials and Methods) and any mutant that grew as a lawn on glucose plates was confirmed by transferring the insertion into a fresh *E. coli* CFT073 background and retesting it for growth on glucose plates (see Materials and Methods).

Five confirmed non-quiescent mini-Tn5 Km mutants were isolated from approximately 2000 mutants tested. *E. coli* CFT073 and the mini-Tn5 Km mutants were grown overnight in liquid glucose M9 minimal medium and viable counts were made on both glucose plates and LB agar plates. As expected, *E. coli* CFT073 assayed from the overnight cultures failed to grow on the glucose plates when $\leq 10^6$ CFU were plated, but when assayed on LB agar viable counts showed that the cultures contained $\sim 10^9$ CFU/ml. In contrast, when assayed on either glucose plates or LB agar plates the 5 mini-Tn5 Km mutant cultures each contained about $\sim 10^9$ CFU/ml. It therefore appears that the mini-Tn5 Km insertion in each of the 5 genes completely prevented quiescence on glucose plates.

The mini-Tn5 Km insertions that resulted in mutants able to grow on glucose plates after transfer into a fresh *E. coli* CFT073 background were in the following 5 genes (Fig. 6): (i) *sdhA*: encodes the succinate-binding flavoprotein subunit of succinate dehydrogenase (27). As a consequence, the *E. coli* CFT073 *sdhA* mutant fails to grow on succinate as sole carbon source, but grows normally on glucose. (ii) *gnd*: encodes 6-phosphogluconate dehydrogenase which functions in the oxidative branch of the pentose phosphate pathway to synthesize ribulose-5-phosphate from 6-phosphogluconate (28, 29). Ribulose-5-phosphate is an essential precursor in the synthesis of FAD, nucleotides, and LPS. (iii) *zwf*: encodes glucose-6-phosphate dehydrogenase, which functions in the oxidative branch of the pentose phosphate pathway and the Entner-Doudoroff pathway when *E. coli* is grown on glucose (28, 29). (iv) *pykF*: encodes pyruvate kinase, which converts phosphoenolpyruvate to pyruvate in the Embden-Meyerhof-Parnas pathway (30). (v) *gdhA*: encodes glutamate dehydrogenase, which catalyzes the amination of α -ketoglutarate to glutamate (31).

It might be argued that the mini-Tn5 Km insertions in the identified genes are not the cause of non-quiescence, but that non-quiescence is caused by downstream polarity effects. Indeed, the mini-Tn5 Km transposon used in the present study has strong transcription termination sequences flanking both ends of the kanamycin resistance gene (17). However, the intergenic number of nucleotides and nucleotide sequences between *gnd* and the immediate downstream *ugd*, between *pykF* and the immediate downstream *lpp*, and between *zwf* and the immediate downstream *edd* are identical in *E. coli* MG1655 and *E. coli* CFT073 (GenBank accession numbers U00096.3 and AE014075.1) (21, 32). Moreover, in *E. coli* MG1655 and therefore in *E. coli* CFT073, there is a strong

presumptive promoter between *gnd* and *ugd* (33) and in *E. coli* MG1655 and therefore in *E. coli* CFT073 both *lpp* and *edd* have their own experimentally identified promoters (33). It is therefore highly likely that non-quiescence is caused by interrupting *gnd*, *pykF*, and *zwf*, and not by downstream polarity effects. Also, in *E. coli* CFT073, *gdhA* is immediately upstream of *c2163*, which is transcribed in the opposite direction to *gdhA* (21), making it highly unlikely that non-quiescence caused by the insertion in *gdhA* is due to downstream polarity. Finally, although in *E. coli* MG1655 the number of nucleotides between the end of the *sdhCDAB* operon and the beginning of the immediately downstream *sucABCD* operon is 241 nucleotides less than that in *E. coli* CFT073 (GenBank accession numbers U00096.3 and AE014075.1) (31, 32), the experimentally identified *E. coli* MG1655 *sucABCD* promoter is identical in sequence to the presumptive *E. coli* CFT073 *sucABCD* promoter and the nucleotide sequence between the 3' end of the promoter and the start of *sucA* transcription is identical in both strains (21, 32, 33). It is therefore highly unlikely that non-quiescence caused by the insertion in *sdhA* is due to a downstream polarity effect on the *sucABCD* operon in *E. coli* CFT073.

Of the 5 mini-Tn5 Km mutants, only *E. coli* CFT073 *sdhA* and *gdhA* generate low levels of persister cells. Since the 5 *E. coli* CFT073 mini-Tn5 Km mutants were non-quiescent on glucose plates, we wondered whether they would generate fewer persister cells than *E. coli* CFT073 in glucose M9 minimal medium. Indeed, the *E. coli* CFT073 *sdhA* and *gdhA* mutants, which began growth shortly after dilution of overnight cultures into fresh glucose M9 minimal medium, generated only about 5×10^2 persister cells/ml, i.e. about 2000-fold fewer persister cells in the presence of ampicillin than wildtype *E. coli* CFT073 (Figs. 7 and 8). In contrast, the *E. coli* CFT073 *gnd*, *pykF*, and *zwf* mutants

generated about the same level of persister cells as *E. coli* CFT073 (Table 4), suggesting that quiescence and persistence are not identical phenomena.

***E. coli* CFT073 persistence and quiescence require a complete TCA cycle.** Succinate dehydrogenase converts succinate to fumarate (Fig. 6). The fact that the *E. coli* CFT073 *sdhA* mutant generated about 2000-fold fewer persister cells than *E. coli* CFT073 suggested the possibility that the ability to make fumarate from succinate was required for persister cell formation. To test that possibility, *E. coli* CFT073 *sdhA* cultures were grown overnight in liquid 0.4% glucose M9 minimal medium in the presence and absence of fumarate (200 µg/ml) and persister cell assays were performed in 0.2% glucose M9 minimal medium in the presence of fumarate for cultures grown with fumarate and in the absence of fumarate for cultures grown without fumarate. In the presence of fumarate and ampicillin, *E. coli* CFT073 *sdhA* cultures generated between 10^5 and 10^6 CFU/ml persister cells (Fig. 9), much like *E. coli* CFT073 (Figs. 4, 5, 7, and 8), whereas in the absence of fumarate and the presence of ampicillin, *E. coli* CFT073 *sdhA* cultures generated only 5×10^2 CFU/ml persister cells (Fig. 9). Therefore, allowing completion of the TCA cycle by supplying *E. coli* CFT073 *sdhA* with fumarate rescued its ability to generate a high level of persister cells, suggesting that a complete oxidative TCA cycle is required for maximal persister cell generation

Since persister cell generation required a complete TCA cycle, we wondered whether the same was true of quiescence. To that end, *E. coli* CFT073 *sdhA* was grown overnight in liquid 0.4% glucose M9 minimal medium in the presence and absence of fumarate (200 µg/ml). Glucose plates containing fumarate (200 µg/ml) were seeded with 10^5 CFU of *E. coli* CFT073 *sdhA* grown in the presence of fumarate or 10^5 CFU of *E. coli* CFT073

sdhA grown in the absence of fumarate and were incubated at 37°C for 24 h. In both cases, *E. coli* CFT073 *sdhA* failed to grow, but responded to 5 µl of a 1.0 mM mixture of lysine, methionine, and tyrosine (Fig. 10A), not only suggesting that the presence of fumarate in the glucose plate rescued quiescence, but that quiescence was generated on the glucose plate and not in the liquid culture. As a control, glucose plates without fumarate were seeded with 10⁵ CFU of *E. coli* CFT073 *sdhA* grown overnight in liquid glucose M9 minimal medium in the absence of fumarate and plates were incubated at 37°C for 24 h. As expected, a lawn of *E. coli* CFT073 *sdhA* growth was observed on the glucose plates under these conditions (Fig. 10B). Therefore, not only does fumarate rescue the ability of *E. coli* CFT073 *sdhA* to generate a high level of persisters in liquid glucose M9 minimal medium in the presence of ampicillin (Fig. 9), it rescues the ability of *E. coli* CFT073 *sdhA* to enter the quiescent state on glucose plates. As a side note, rescue by fumarate, obviating the need for succinate dehydrogenase, further implicates the mini-Tn5 Km insertion and not a downstream polarity effect as the cause of non-quiescence and reduced persistence of the *sdhA* mutant.

The *E. coli* CFT073 original clinical isolate is quiescent on glucose plates, but generates very few persister cells in liquid glucose M9 minimal medium. As we were completing this study, we became aware of a paper reporting that the sequenced *E. coli* CFT073 (22), which has long been considered to be the wildtype strain, has a 5 bp duplication in *rpoS* which results in a truncated, non-functional RpoS (34). RpoS, often referred to as the alternative sigma factor σ^S , directs RNA polymerase to transcribe genes involved in the *E. coli* general stress response, e.g. acid resistance (35). The *E. coli* CFT073 used in this study does indeed have the 5 bp duplication in *rpoS* (Leatham-

Jensen MP, unpublished results). We therefore obtained and tested the original clinical isolate of *E. coli* CFT073, which has a wildtype *rpoS* gene (34), for quiescence and persister cell formation. The *E. coli* CFT073 original clinical isolate is indeed quiescent on glucose plates and responds to lysine, methionine, and tyrosine like the *rpoS* mutant used here (Fig. 11), but generates about 2000-fold fewer persister cells in liquid glucose M9 minimal medium than the *rpoS* mutant used in the present study (Table 4). Therefore, it appears that the mutant *rpoS* gene is necessary for the high level of persistence observed, but is not necessary for *E. coli* CFT073 quiescence on glucose plates.

Discussion

The data presented here show that the uropathogen *E. coli* CFT073 and the probiotic *E. coli* Nissle 1917, both ST73 strains belonging to phylogenetic group B2, are quiescent on glucose plates seeded with $\leq 10^6$ CFU, as are 35/45 (77.8%) additional ST73 strains tested. ST73 is a very common UPEC lineage (24, 25). In contrast, of 4 phylogenetic group A, 6 phylogenetic group B1, 6 phylogenetic group D, 4 phylogenetic group ABD, and 4 phylogenetic group AxB1 strains, none (0/24) were quiescent. However, 9 of 40 randomly selected UPEC strains isolated from community acquired UTIs in Denmark (22.5%) were quiescent on glucose plates (3/5 ST73 strains, 3/3 ST141 strains, 1/1 ST104 strain, 1/1 ST394 strain, and 1/1 ST998 strain). Thus quiescence on glucose plates is common among UPEC isolates and is not restricted to one ST type.

The data presented here also show that the *E. coli* CFT073 original clinical isolate, like the *E. coli* CFT073 strain used here, which has a 5 bp duplication in *rpoS* that inactivates the gene (33), is quiescent on glucose plates (Fig. 11), but unlike the *E. coli*

CFT073 strain used here, generates a low level of persister cells in the presence of ampicillin (Table 4). Therefore, quiescence and persistence, while similar in some respects, i.e. both are prevented by amino acids and both require a complete oxidative TCA cycle, are not identical. In addition, quiescence and persistence differ in that of the 5 *E. coli* CFT073 mini-Tn5 non-quiescent mutants isolated in the present study (*gdhA*, *gnd*, *pykF*, *sdhA*, and *zwf*), only 2 (*gdhA* and *sdhA*) were deficient in persister cell formation (Table 4).

It has been reported, as reported here for *E. coli* CFT073, that deleting *rpoS* in *E. coli* K-12 also dramatically increases formation of persister cells in the presence of ampicillin (36). Why then, does *E. coli* Nissle 1917, which has a wildtype *rpoS* gene (Leatham-Jensen MP, unpublished results and [36]), generate at least as high a level of persister cells as *E. coli* CFT073 (Table 4)? *E. coli* Nissle 1917 would be expected to be acid resistant since a wildtype *rpoS* gene is required for acid resistance; however, it has been reported to be extremely acid sensitive (37). This suggests the possibility that the *E. coli* Nissle 1917 *rpoS* gene may be poorly expressed or that RpoS is rapidly degraded, which is consistent with its generating as high a level of persister cells as the *E. coli* CFT073 *rpoS* mutant used here. Many *E. coli* strains contain wildtype *rpoS* genes that are expressed poorly relative to other strains (38).

While it is unclear as to why *E. coli* CFT073 *gnd* and *zwf* mutants are non-quiescent on glucose plates, the reason *E. coli* K-12 *gnd* and *zwf* mutants grow on glucose as sole carbon source is known (29, 30). When *E. coli* K-12 is grown on glucose as sole carbon source, glucose-6-phosphate dehydrogenase, encoded by *zwf*, and 6-phosphogluconate dehydrogenase, encoded by *gnd*, are used for the synthesis of ribulose-5-phosphate via

the oxidative branch of the pentose phosphate pathway (Fig. 6 and [28, 29]). Ribulose-5-phosphate is an essential precursor in the synthesis of FAD, nucleotides, and LPS. Furthermore, both enzymes generate NADPH for biosynthesis. It might therefore seem surprising that mutations in *gnd* and *zwf* allow growth on glucose as sole carbon source (Fig. 6). However, null mutations in *gnd* and *zwf* in *E. coli* K-12 do not significantly affect their growth on glucose because the non-oxidative branch of the pentose phosphate pathway runs backwards in these mutants, generating ribulose-5-phosphate from fructose-6-phosphate and glyceraldehyde-3-phosphate (Fig. 6 and [28, 29]) and the necessary NADPH for biosynthesis by increased flux through the TCA cycle (28, 29). It is therefore possible that for unknown mechanistic reasons the oxidative branch of the pentose phosphate pathway operates minimally during *E. coli* CFT073 quiescence, generating little ribulose-5-phosphate, but that the *gnd* and *zwf* mutations cause the non-oxidative branch to run backwards and flux through the TCA cycle to increase, generating a sufficient level of ribulose-5-phosphate and NADPH to prevent quiescence. This scenario is consistent with the observation that ribose and xylose, both of which are metabolized in the non-oxidative branch of the pentose phosphate pathway, prevent quiescence when used as sole carbon sources (Fig. 2).

The *E. coli* CFT073 *pykF* mutant also grows on glucose as sole carbon source. The *pykF* gene encodes pyruvate kinase which converts PEP to pyruvate (Fig. 6). There is a second pyruvate kinase, encoded by *pykA*, but during *E. coli* K-12 growth in glucose minimal medium, it contributes little to total pyruvate kinase activity (39). Moreover, in the complete absence of pyruvate kinase, pyruvate can still be generated in *E. coli* K-12 both through glucose transport via the phosphotransferase transport system (PTS) (40)

and the Entner-Doudoroff pathway (31, 38). Therefore, it is not surprising that the non-quiescent *E. coli* CFT073 *pykF* mutant can grow on glucose as sole carbon source, but why is it non-quiescent? It has been suggested that *pykF* mutants contain increased intracellular levels of PEP (30). If PEP, a common precursor in lysine, methionine, and tyrosine biosynthesis (Fig. 6), is increased in the *E. coli* CFT073 *pykF* mutant growing on glucose plates, sufficient intracellular levels of the 3 amino acids might be generated to stimulate growth and prevent quiescence.

The *gdhA* gene encodes glutamate dehydrogenase, which converts α -ketoglutarate to glutamate (Fig. 6). It is known that *E. coli* K-12 *gdhA* mutants grow on glucose as sole carbon source in the absence of glutamate dehydrogenase because glutamate can also be synthesized via the sequential action of glutamine synthetase and glutamate synthase (31, 41). Therefore, it appears that the switch from producing glutamate via glutamate dehydrogenase to producing it via glutamine synthetase and glutamate synthase in *E. coli* CFT073 somehow prevents quiescence. It has been estimated that 88% of assimilated nitrogen in *E. coli*, including the nitrogen in amino acids, originates from glutamate and the remaining 12% from glutamine (42). Perhaps glutamate and glutamine are in higher concentrations in *gdhA* mutants growing on glucose, which could contribute to higher intracellular levels of lysine, methionine, and tyrosine and as a consequence, non-quiescence.

E. coli K-12 succinate dehydrogenase mutants grow on glucose as sole carbon source because it is not necessary for the TCA cycle to function as a complete oxidative cycle to achieve growth (43). In fact, when growing on excess glucose, the *E. coli* K-12 TCA cycle operates in branched mode, i.e. an oxidative branch which runs from citrate to α -

ketoglutarate and a reductive branch which runs backwards from oxaloacetate to succinyl-CoA (Fig. 6 and [43]). Succinate dehydrogenase is not necessary under these conditions and both branches serve biosynthetic functions. As a result, neither branch is used for ATP generation (42). ATP is generated from glycolysis and via the phosphotransacetylase (*pta*)-acetate kinase (*ackA*) pathway producing acetate in the process (Fig. 6 and [43]). Therefore, it appears likely that forcing the TCA cycle to operate in branched mode somehow prevents *E. coli* CFT073 quiescence. In branched mode, the TCA cycle is unable to regenerate oxaloacetate and therefore gets oxaloacetate from PEP via PpC (Fig. 6). Perhaps under these conditions sufficient oxaloacetate is generated via PpC to increase intracellular levels of lysine and methionine (Fig. 6) and consequently, prevent quiescence. In this vein, addition of fumarate to glucose plates, results in return of the *E. coli* CFT073 *sdhA* mutant to quiescence, presumably by rescuing the ability of the TCA cycle to operate as a complete oxidative cycle. It should also be noted that an *E. coli* CFT073 succinate dehydrogenase mutant (*sdhB*) has been shown to be severely attenuated in an ascending mouse UTI model (44), indicating a possible link between in vitro non-quiescence and reduced pathogenesis in vivo.

While it is clear why the *E. coli* CFT073 mini-Tn5 Km non-quiescent mutants are capable of growth using glucose as sole carbon source, it is not mechanistically clear as to why the mutations prevent quiescence on glucose plates. Possibly, a gene encoding a regulator whose synthesis or activity is inhibited by various combinations of lysine, methionine, and tyrosine, promotes quiescence. The regulator would presumably be expressed or active in the vast majority of *E. coli* CFT073 cells on glucose plates, but in relatively few cells of the *E. coli* CFT073 mini-Tn5 Km non-quiescent mutants due to the

complex metabolic changes that occur in such mutants (28, 29, 30, 39, 45, 46). If expression and activity of a specific *E. coli* CFT073 regulator is critical for generation of quiescence, much as toxin/antitoxin systems appear to be critical in generating persister cells (11, 47), screening more mini-Tn5 Km mutants may lead to its identification. Furthermore, it is unclear as to why the *E. coli* CFT073 *gdhA* and *sdhA* mutants generate far fewer persister cells than the *gnd*, *zwf*, and *pykF* mutants (Table 4). Metabolic flux analysis (28, 29, 30) and RNA-seq (48) may prove useful in this regard.

How might the findings reported here be relevant to recurrent UTI infections and how might its relevance be tested? It is known that *E. coli* CFT073 utilizes amino acids and small peptides as carbon sources and a complete oxidative TCA cycle to infect the mouse urinary tract (44, 49) and appears to import small peptides to grow in mouse urine in vitro (44). UPEC may also use peptides for growth in vivo in urine during human UTI (50). It is also known that *E. coli* CFT073 can infect mouse superficial facet cells and form biofilm-like intracellular communities (IBCs) (51) and therefore most likely can form **Quiescent Intracellular Reservoirs (QIRs)** in underlying transitional cells. Furthermore, it seems reasonable that QIRs surviving antibiotic treatment serve as a reservoir for recurrent UTI infection after withdrawal of antibiotics (8, 10), i.e. as transitional cells undergo apoptosis and released QIRs resume growth in urine using peptides and amino acids in the process (44, 49, 50). However, it should be noted that the role of QIRs in recurrent UTI is still controversial. It has recently been shown that UPEC strains isolated from the feces and urine of female patients during recurrent episodes are identical, consistent with the possibility that recurrent UTI is caused by UPEC strains that colonize the intestine, but periodically move to the urinary tract (52). Nevertheless, if the in vitro

quiescence reported here mimics the QIR state and if QIRs are a major source of recurrent UTI, the *E. coli* CFT073 non-quiescent mutants we've isolated should be less able to establish QIRs in the mouse bladder and therefore be less able to cause recurrent UTI. If so, it is possible that drugs designed to inactivate the enzymes encoded by *gdhA*, *gnd*, *pykF*, *sdhA*, and *zwf* might be effective in limiting recurrent urinary tract infection. On the other hand, if the in vitro persistence state mimics the QIR state in mouse bladder cells, only the *E. coli* CFT073 *gdhA* and *sdhA* mutants should be less able to cause recurrent UTI and if so, drugs designed to inactivate the enzymes encoded by *gdhA* and *sdhA* might be effective in limiting recurrent urinary tract infection. A recurrent UTI mouse model is available for testing these hypotheses (8).

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Table 1. Bacterial Strains

<u><i>E. coli</i> strain</u>	<u>Genotype/phenotype</u>	<u>Referred to in text as:</u>	<u>Source/Reference</u>
CFT073 Str ^R	Spontaneous streptomycin resistant mutant of CFT073, has 5 bp duplication in <i>rpoS</i>	CFT073	(53)
CFT073 Str ^R mini- Tn5 Km:: <i>gdhA</i>	mini-Tn5 Km glutamate dehydrogenase mutant of CFT073 Str ^R	CFT073 <i>gdhA</i>	This study
CFT073 Str ^R mini- Tn5 Km:: <i>gnd</i>	mini-Tn5 Km 6-phosphogluconate dehydrogenase mutant of CFT073 Str ^R	CFT073 <i>gnd</i>	This study
CFT073 Str ^R mini- Tn5 Km:: <i>pykF</i>	mini-Tn5 Km pyruvate kinase mutant of CFT073 Str ^R	CFT073 <i>pykF</i>	This study
CFT073 Str ^R mini- Tn5 Km:: <i>sdhA</i>	mini-Tn5::Km flavoprotein subunit of succinate dehydrogenase	CFT073 <i>sdhA</i>	This study

	mutant of CFT073 Str ^R		
CFT073 Str ^R mini-Tn5 Km:: <i>zwf</i>	mini-Tn5::Km glucose-6-phosphate dehydrogenase mutant of CFT073 Str ^R	CFT073 <i>zwf</i>	This study
wildtype CFT073	original clinical isolate	CFT073 original clinical isolate	(34)
Nissle 1917 Str ^R	spontaneous streptomycin resistant mutant of Nissle 1917	Nissle 1917	(57)
MG1655 Str ^R	spontaneous streptomycin resistant mutant of MG1655	MG1655	(54)
HS Str ^R	spontaneous streptomycin resistant mutant of HS	HS	(55)
EFC1 Str ^R	spontaneous streptomycin resistant mutant of EFC1	EFC1	(55)

EFC2 Str ^R	spontaneous streptomycin resistant mutant of EFC2	EFC2	(55)
F-18 Str ^R Nal ^R	spontaneous streptomycin and nalidixic acid resistant mutant of F-18	F-18	(56)
EDL933 Str ^R	spontaneous streptomycin resistant mutant of EDL933	EDL933	(54)
ATM161	host for pUT, which contains the mini- Tn5 Km transposon, resistant to kanamycin	ATM161	(17)

Table 2. PCR primer sequences for amplifying mutant genes containing mini-Tn5 Km insertions.

gene	Primer 1: 5'→3'	Primer 2: 5'→3'
<i>gdh</i> <i>A</i>	GATGGTCGAGTGGCAGATTAC	CAGAGGCTACTCAATGGCTT AC
<i>gnd</i>	GTTGGTTAAATCAGATTAATCCAGCC	CAACAGATCGGCGTAGTCG
<i>pykF</i>	CTGTAGCAATTGAGCGATGAT G	ATCAGGGCGCTTCGATATAC
<i>sdhA</i>	CCGTTCCCATAACGTTTCTG	TTTCACCGGATCAACGTGAG
<i>zwf</i>	CCGGTAAAATAACCATAAAGGATAA GC	GAGAATGACATGGCGGTAAC

Table 3. Free amino acid composition of 50-fold concentrated *E. coli* MG1655 and *E. coli* CFT073 supernatants and human urine (μM).

Amino Acid ^a	<i>E. coli</i> MG1655 Supernatant ^b	<i>E. coli</i> CFT073 Supernatant ^b	Human Urine ^c
alanine	353	383	3350
arginine	-	-	205
aspartic acid	-	22	-
cysteine	-	-	1110
glutamic acid	360	848	-
glycine	11	86	21200
histidine	-	-	9470
isoleucine	90	170	478
leucine	56	149	382
lysine	7472	4059	4480
methionine	59	37	171
phenylalanine	99	187	626
proline	144	116	-
serine	64	70	4000
threonine	201	461	2430
tryptophan	146	312	-
tyrosine	13	52	1060
valine	229	748	349

^a amino acids not listed are not present in the preparations; ^b see Materials and Methods for details; ^c average of 39 women, reference (26).

Table 4. *E. coli* CFT073 persister cells relative to persister cells for other *E. coli* strains.

<i>E. coli</i> strain	# of experiments	% persister cells \pm SEM ^a	CFT073 persister cells relative to: ^a	<i>P</i> value
CFT073	7	0.71 \pm 0.19	-	
CFT073 <i>gnd</i>	2	0.34 \pm 0.18	1.65	0.44
CFT073 <i>pykF</i>	2	2.37 \pm 0.18	0.23	0.075
CFT073 <i>zwf</i>	2	0.29 \pm 0.29	1.45	0.43
Nissle 1917	2	2.64 \pm 1.22	0.21	0.16
True wildtype CFT073	2	2.80 $\times 10^{-4} \pm 1.1 \times 10^{-4}$	1160	0.025*

^a % persister cells was calculated by dividing the viable count at 24 h by the viable count at time zero times 100. The value for *E. coli* CFT073 persister cells relative to a specific *E. coli* strain is calculated by dividing the % persister cells at 24 h generated by *E. coli* CFT073 in the experiments listed for the specific strain by the % persister cells at 24 h generated by that specific strain in those experiments. * $P < 0.05$ using the two-tailed Student t test is considered to be statistically significant.

Legends to the Figures

Fig. 1. *E. coli* CFT073 quiescence on glucose plates. A 0.2% glucose plate was seeded with 10^5 CFU of *E. coli* CFT073 (see Materials and Methods). (A), 60 min after seeding the plate, a colony of *E. coli* MG1655, grown on a glucose plate, was toothpicked to the plate seeded with *E. coli* CFT073, which was then incubated at 37°C for 24 h; (B), the same plate, incubated for 48 h. Note that *E. coli* CFT073 only grows around the toothpicked *E. coli* MG1655. Although not shown, *E. coli* Nissle 1917 undergoes quiescence on glucose plates identically.

Fig. 2. *E. coli* CFT073 non-quiescence on glycerol, ribose, and xylose plates. 0.2% glucose, glycerol, ribose, and xylose plates were seeded with 10^5 CFU of *E. coli* CFT073 grown overnight in liquid M9 minimal medium containing their respective sugars (0.4%). 60 min after seeding the plates, a colony of *E. coli* MG1655, grown on a glucose plate, was toothpicked to the glucose plate. Plates were incubated at 37°C for 24h. (A), glycerol plate; (B), ribose plate; (C), xylose plate; (D), glucose plate.

Fig. 3. Prevention of quiescence by human urine and amino acids. 0.2% glucose plates were seeded with 10^5 CFU of *E. coli* CFT073 and 5 µl of the following mixtures were spotted onto the plates. (A), 50-fold concentrated *E. coli* MG1655 culture supernatant; (B), amino acid cocktail mimicking amino acid concentrations in the 50-fold concentrated *E. coli* MG1655 culture supernatant; (C), human urine; (D), amino acid cocktail mimicking amino acid concentrations in human urine (Table 3); (E), lysine, methionine, tyrosine (1.0 mM each); (F), lysine and methionine (1.0 mM each); (G), lysine and tyrosine (1.0 mM each); (H), methionine and tyrosine (1.0 mM each). Plates

were incubated at 37°C for 24h. Although not shown, results for *E. coli* Nissle 1917 were essentially identical.

Fig. 4. *E. coli* CFT073 and *E. coli* MG1655 persistence. Cultures were grown overnight in

0.4 % glucose M9 minimal medium as described in Material and Methods. Persister cell assays were performed as described in Materials and Methods. (▲), *E. coli* CFT073; (●), *E. coli* CFT073 plus ampicillin; (◆), *E. coli* MG1655; (■), *E. coli* MG1655 plus ampicillin. Bars representing standard errors of the log₁₀ means of CFU per ml for 2 independent experiments are presented for each time point. At 24 h the approximate 1000-fold difference between *E. coli* CFT073 persisters in the presence of ampicillin and *E. coli* MG1655 persisters in the presence of ampicillin is statistically significant (P = 0.0052).

Fig. 5. *E. coli* CFT073 persistence in the presence of amino acids. Cultures were grown in 0.4 % glucose M9 minimal medium as described in Material and Methods and diluted 20-fold into 0.2 % glucose M9 minimal medium either containing or lacking a mixture of the 20 standard L-amino acids, each at 100 µg/ml and containing or lacking ampicillin (100 µg/ml). (◆), *E. coli* CFT073; (■), *E. coli* CFT073 plus ampicillin; (▲), *E. coli* CFT073 plus amino acids; (●), *E. coli* CFT073 plus amino acids plus ampicillin. Bars representing standard errors of the log₁₀ means of CFU per ml for 2 independent experiments are presented for each time point. At 6 h and 24 h the approximate 100-fold differences between *E. coli* CFT073 persisters in the presence of ampicillin and *E. coli*

MG1655 persisters in the presence of ampicillin are statistically significant ($P = 0.002$ and $P = 0.05$ respectively).

Fig. 6. Diagram of *E. coli* central carbon metabolism. Arrows indicate the physiological directions of the reactions. Genes encoding the enzymes for each reaction are listed beside each reaction. Mini-Tn5 Km insertions in *E. coli* CFT073 genes that result in non-quiescence on glucose plates are circled.

Fig. 7. *E. coli* CFT073 *sdhA* persistence. Cultures were grown overnight in 0.4 % glucose M9 minimal medium and persister cell assays were performed as described in Materials and Methods. (♦), *E. coli* CFT073; (■), *E. coli* CFT073 plus ampicillin; (▲), *E. coli* CFT073 *sdhA*; (●), *E. coli* CFT073 *sdhA* plus ampicillin. Bars representing standard errors of the \log_{10} means of CFU per ml for 4 independent experiments are presented for each time point. At 24 h the approximate 2000-fold difference between *E. coli* CFT073 persisters in the presence of ampicillin and *E. coli* CFT073 *sdhA* persisters in the presence of ampicillin is statistically significant ($P < 0.001$).

Fig. 8. *E. coli* CFT073 *gdhA* persistence. Cultures were grown overnight in 0.4 % glucose M9 minimal medium and persister cell assays were performed as described in Materials and Methods. (♦), *E. coli* CFT073; (■), *E. coli* CFT073 plus ampicillin; (▲), *E. coli* CFT073 *gdhA*; (●), *E. coli* CFT073 *gdhA* plus ampicillin. Bars representing standard errors of the \log_{10} means of CFU per ml for 3 independent experiments are presented for each time point. At 24 h the approximate 2000-fold difference between *E. coli* CFT073 persisters in the presence of ampicillin and *E. coli* CFT073 *gdhA* persisters in the presence of ampicillin is statistically significant ($P < 0.001$).

Fig. 9. *E. coli* CFT073 *sdhA* persistence in the presence of fumarate. Cultures were grown overnight in 0.4 % glucose M9 minimal medium containing or lacking disodium fumarate (200 µg/ml), diluted 20-fold into 0.2% glucose M9 minimal medium containing or lacking fumarate, and persister cell assays were performed as described in Materials and Methods. (▲), *E. coli* CFT073 *sdhA*; (●), *E. coli* CFT073 *sdhA* plus ampicillin; (◆), *E. coli* CFT073 *sdhA* plus fumarate; (■), *E. coli* CFT073 *sdhA* plus fumarate plus ampicillin. Bars representing standard errors of the log₁₀ means of CFU per ml for 2 independent experiments are presented for each time point. At 6 h and 24 h the differences between *E. coli* CFT073 *sdhA* persisters in the presence and absence of fumarate are statistically significant ($P = 0.013$ and $P = 0.046$ respectively).

Fig. 10. Rescue of *E. coli* CFT073 *sdhA* quiescence by fumarate. *E. coli* CFT073 *sdhA* was grown overnight in 0.4 % glucose M9 minimal medium and 10⁵ CFU were seeded on (A), 0.2% glucose plates containing disodium fumarate (200 µg/ml) or (B), 0.2% glucose plates. One hour later, 5 µl of a mixture of 1.0 mM lysine, 1.0 mM methionine, and 1.0 mM tyrosine was spotted to each plate. Plates were incubated at 37°C for 24 h.

Fig. 11. Quiescence of the *E. coli* CFT073 original clinical isolate on glucose plates. 0.2% glucose plates were seeded with 10⁵ CFU of the *E. coli* CFT073 original clinical isolate and 5 µl of the following mixtures (1.0 mM of each amino acid) were spotted onto the plates. (A), lysine, methionine, tyrosine; (B), lysine and methionine; (C), lysine and tyrosine; (D), methionine and tyrosine. Plates were incubated at 37°C for 24h.

Figure 1

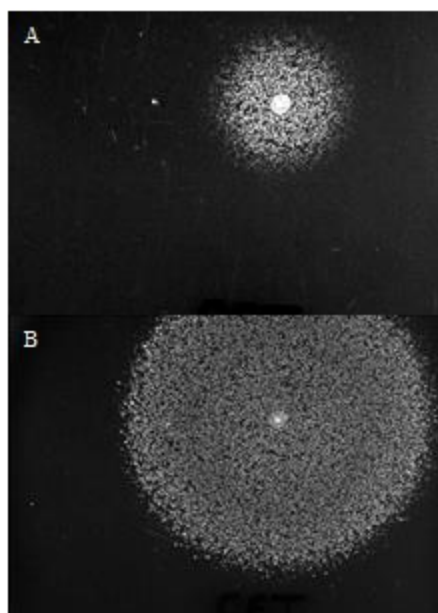


Fig. 1. *E. coli* CFT073 quiescence on glucose plates. A 0.2% glucose plate was seeded with 10^6 CFU of *E. coli* CFT073 (see Materials and Methods). (A), 60 min after seeding the plate, a colony of *E. coli* MG1655, grown on a glucose plate, was toothpicked to the plate seeded with *E. coli* CFT073, which was then incubated at 37°C for 24 h; (B), the same plate, incubated for 48 h. Note that *E. coli* CFT073 only grows around the toothpicked *E. coli* MG1655. Although not shown, *E. coli* Nissle 1917 undergoes quiescence on glucose plates identically.

Figure 2

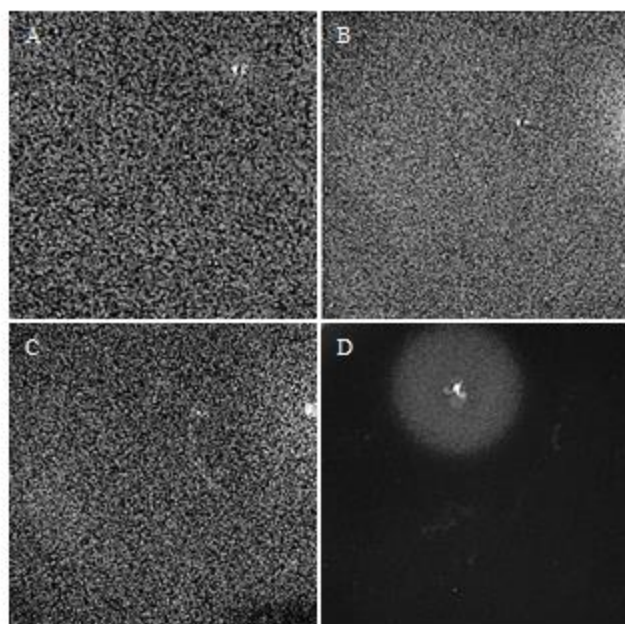


Fig. 2. *E. coli* CFT073 non-quiescence on glycerol, ribose, and xylose plates. 0.2% glucose, glycerol, ribose, and xylose plates were seeded with 10^4 CFU of *E. coli* CFT073 grown overnight in liquid M9 minimal medium containing their respective sugars (0.4%). 60 min after seeding the plates, a colony of *E. coli* MG1655, grown on a glucose plate, was toothpicked to the glucose plate. Plates were incubated at 37° C for 24 h. (A), glycerol plate; (B), ribose plate; (C), xylose plate; (D), glucose plate.

Figure 3

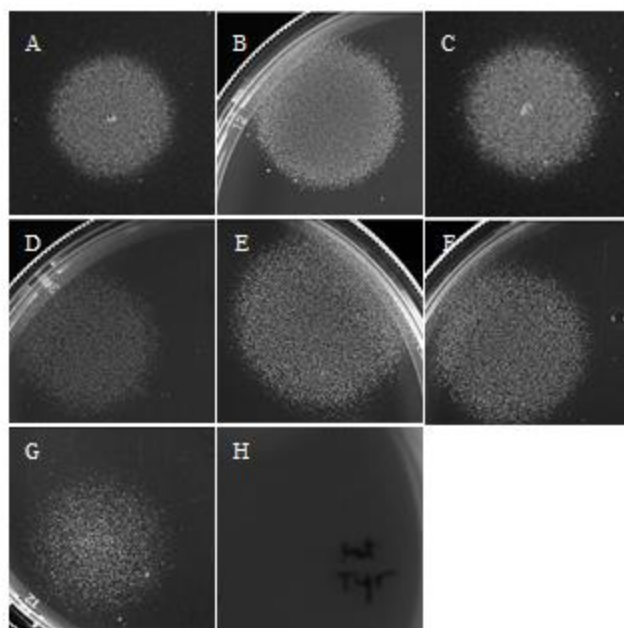


Fig. 3. Prevention of quiescence by human urine and amino acids. 0.2% glucose plates were seeded with 10^8 CFU of *E. coli* CFT073 and 5 μ l of the following mixtures were spotted onto the plates. (A), 50-fold concentrated *E. coli* MG1655 culture supernatant; (B), amino acid cocktail mimicking amino acid concentrations in the 50-fold concentrated *E. coli* MG1655 culture supernatant; (C), human urine; (D), amino acid cocktail mimicking amino acid concentrations in human urine (Table 3); (E), lysine, methionine, tyrosine (1.0 mM each); (F), lysine and methionine (1.0 mM each); (G), lysine and tyrosine (1.0 mM each); (H), methionine and tyrosine (1.0 mM each). Plates were incubated at 37° C for 24h. Although not shown, results for *E. coli* Nissle 1917 were essentially identical.

Figure 4

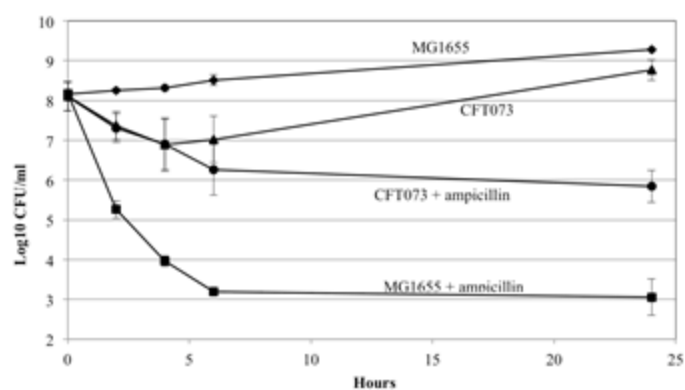


Fig. 4. *E. coli* CFT073 and *E. coli* MG1655 persistence. Cultures were grown overnight in 0.4 % glucose M9 minimal medium as described in Materials and Methods. Persister cell assays were performed as described in Materials and Methods. (▲), *E. coli* CFT073; (●), *E. coli* CFT073 plus ampicillin; (◆), *E. coli* MG1655; (■), *E. coli* MG1655 plus ampicillin. Bars representing standard errors of the log₁₀ means of CFU per ml for 2 independent experiments are presented for each time point.

Figure 5

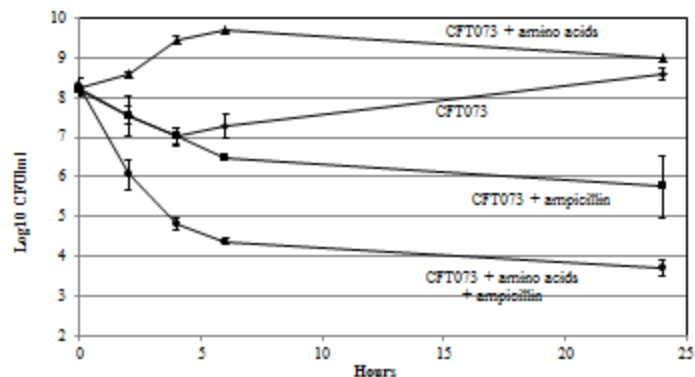


Fig. 5. *E. coli* CFT073 persistence in the presence of amino acids. Cultures were grown in 0.4 % glucose M9 minimal medium as described in Material and Methods and diluted 20-fold into 0.2 % glucose M9 minimal medium either containing or lacking a mixture of the 20 standard L-amino acids, each at 100 μ g/ml and containing or lacking ampicillin (100 μ g/ml). (\blacklozenge), *E. coli* CFT073; (\blacksquare), *E. coli* CFT073 plus ampicillin; (\blacktriangle), *E. coli* CFT073 plus amino acids; (\bullet), *E. coli* CFT073 plus amino acids plus ampicillin. Bars representing standard errors of the \log_{10} means of CFU per ml for 2 independent experiments are presented for each time point.

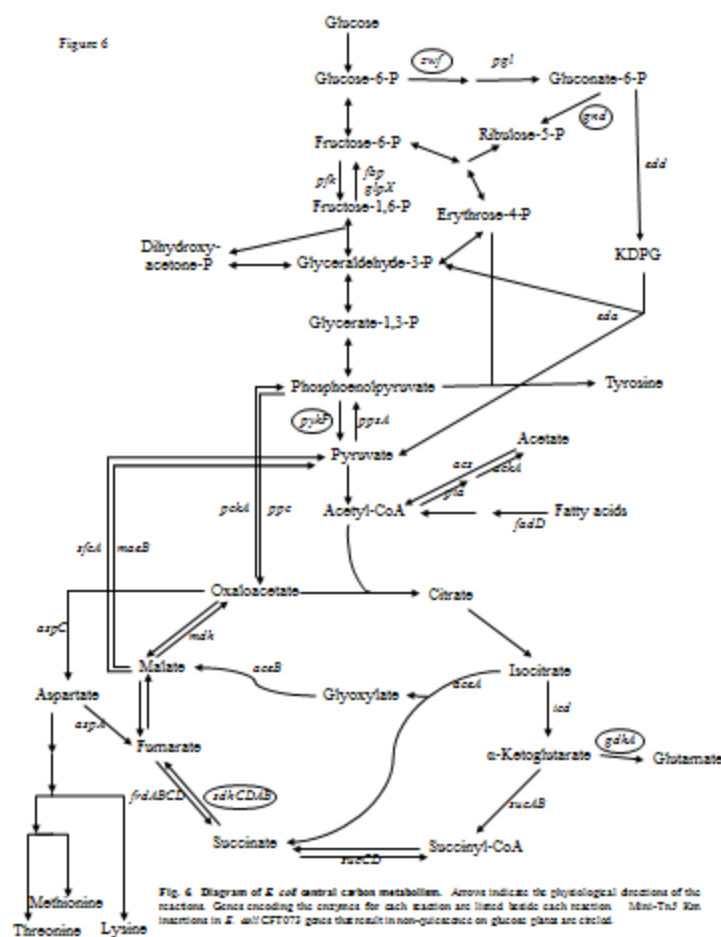


Figure 7

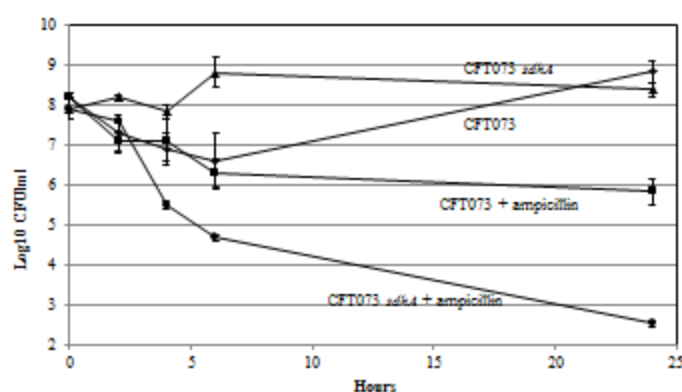


Fig. 7. *E. coli* CFT073 $\Delta sk4$ persistence. Cultures were grown overnight in 0.4% glucose M9 minimal medium and persister cell assays were performed as described in Materials and Methods. (♦), *E. coli* CFT073; (■), *E. coli* CFT073 plus ampicillin; (▲), *E. coli* CFT073 $\Delta sk4$; (●), *E. coli* CFT073 $\Delta sk4$ plus ampicillin. Bars representing standard errors of the log₁₀ means of CFU per ml for 2 independent experiments are presented for each time point.

Figure 8

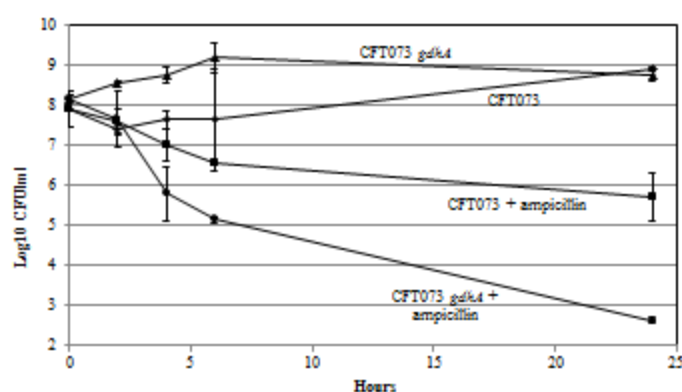


Fig. 8. *E. coli* CFT073 *gdhA* persistence. Cultures were grown overnight in 0.4 % glucose M9 minimal medium and persister cell assays were performed as described in Materials and Methods. (◆), *E. coli* CFT073; (■), *E. coli* CFT073 plus ampicillin; (▲), *E. coli* CFT073 *gdhA*; (●), *E. coli* CFT073 *gdhA* plus ampicillin. Bars representing standard errors of the log₁₀ means of CFU per ml for 2 independent experiments are presented for each time point.

Figure 9

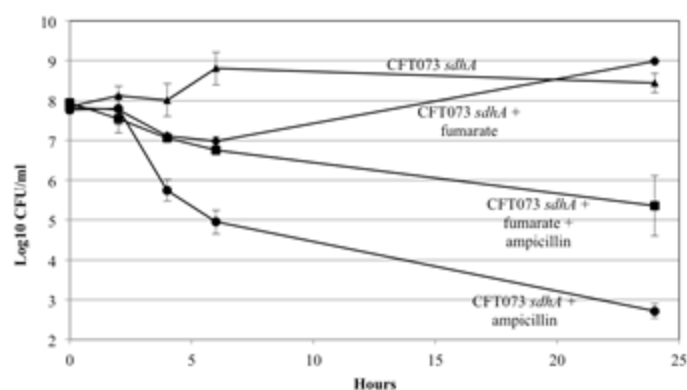


Fig. 9. *E. coli* CFT073 *sdiA* persistence in the presence of fumarate. Cultures were grown overnight in 0.4 % glucose M9 minimal medium containing or lacking disodium fumarate (200 μ g/ml), diluted 20-fold into 0.2% glucose M9 minimal medium containing or lacking fumarate, and persister cell assays were performed as described in Materials and Methods. (▲), *E. coli* CFT073 *sdiA*; (●), *E. coli* CFT073 *sdiA* plus ampicillin; (◆), *E. coli* CFT073 *sdiA* plus fumarate; (■), *E. coli* CFT073 *sdiA* plus fumarate plus ampicillin. Bars representing standard errors of the log₁₀ means of CFU per ml for 2 independent experiments are presented for each time point.

Figure 10

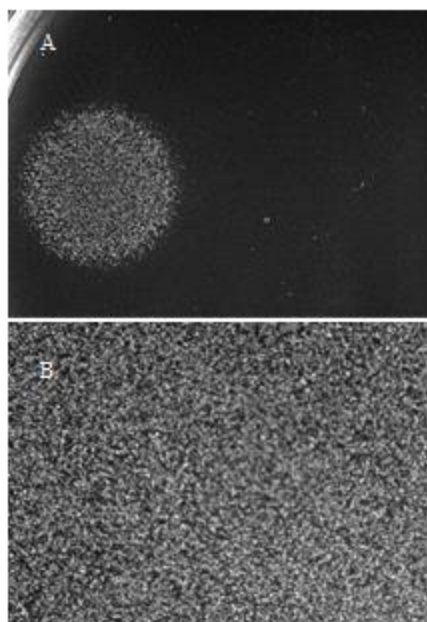


Fig. 10. Rescue of *E. coli* CFT073 *adhA* quiescence by fumarate. *E. coli* CFT073 *adhA* was grown overnight in 0.4 % glucose M9 minimal medium and 10^8 CFU were seeded on (A), 0.2% glucose plates containing disodium fumarate (200 µg/ml) or (B), 0.2% glucose plates. One hour later, 5 µl of a mixture of 1.0 mM lysine, 1.0 mM methionine, and 1.0 mM tyrosine was spotted to each plate. Plates were incubated at 37° C for 24 h.

Figure 11

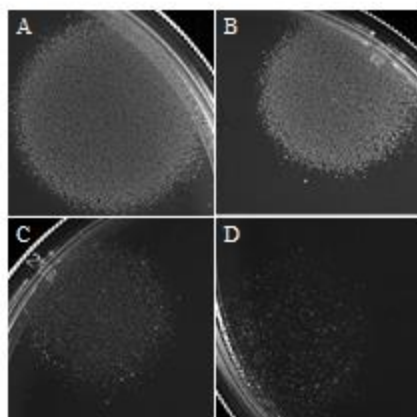


Fig. 11. Quiescence of the *E. coli* CFT073 original clinical isolate on glucose plates. 0.2% glucose plates were seeded with 10^8 CFU of the *E. coli* CFT073 original clinical isolate and 5 μ l of the following mixtures (1.0 mM of each amino acid) were spotted onto the plates. (A), lysine, methionine, tyrosine; (B), lysine and methionine; (C), lysine and tyrosine; (D), methionine and tyrosine. Plates were incubated at 37° C for 24h.

CHAPTER 5

This manuscript is formatted for publication in Scientific Reports. The formatting follows the guidelines set forth by the journal.

Constituents from Cranberry Juice Prevent Quiescence and Persistence in Uropathogenic
Escherichia coli

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Abstract

Urinary tract infections (UTIs) caused by *Escherichia coli* create a large burden on healthcare and frequently cause recurrent infections. Contributing to the success of *E. coli* as an uropathogenic bacterium is its ability to form quiescent intracellular reservoirs in bladder cells and persistence after antibiotic treatment. Cranberry juice and related products have been used extensively in the treatment and prevention of UTIs with varying degrees of benefit reported in the literature. In this study, cranberry oligosaccharides and related compounds were found to both reduce the fraction of persister cells formed and inhibit quiescence in the uropathogenic strain, CFT073. This is the first report detailing components of cranberry juice with the ability to modulate these important physiological aspects of uropathogenic *E. coli*, and further studies investigating cranberry juice should be keen to include oligosaccharides as part of the ‘active’ cocktail of chemical compounds.

Bacterial urinary tract infections (UTIs) are among the most common infections in the world affecting up to 150 million people each year.¹ In the US alone, it is estimated that the total societal cost burden of UTIs is \$3.5 billion each year.² Women are one of the most important at risk demographics with a lifetime prevalence of over 50% and an estimated recurrence of about 25%.³ While UTIs are commonly mild and curable, recurrent infections can require continuous antibiotic prophylaxis for prevention which can lead to adverse events such as colitis, the development of antibiotic resistant UTIs, or the development of complicated UTIs.²⁻⁴

Uropathogenic *Escherichia coli* (UPEC) is the causative pathogen in the majority of UTIs, with estimates ranging anywhere from 65-90% of cases.^{2,4-6} When UPEC is the identified pathogen in a recurrent UTI, the original infecting UPEC strain is responsible for the recurrence 77% of the time.⁷ While the mechanism of recurrence is not fully understood, it is thought that intracellular biofilm-like communities (IBCs) and quiescent intracellular reservoirs (QIRs) contribute significantly to recurrent UTIs.⁸⁻¹⁰ During acute infection, UPEC are able to adhere to, invade, and replicate inside of superficial bladder cells, forming IBCs.^{6,10} As the infection progresses, UPEC infection of the urothelial transitional cells can lead to establishment of QIRs which can remain viable for months intracellularly.^{6,9,11} Importantly, it is also thought that QIRs may not be affected by antibiotics, contributing to treatment failures and recurrence.^{8,10} Upon exfoliation of the bladder epithelial cells containing the QIRs, the UPEC would be re-exposed to the urine and could cause recurrent infection.⁶ It was also shown *in vitro* that the amino acid content of the urine is able to reverse quiescence in UPEC, and could serve as the necessary signal for reinfection.¹²

A similar physiologic state to quiescence in UPEC is bacterial persistence.¹³⁻¹⁶ Persister cells are a small subpopulation of bacteria in a dormant, non-dividing state characterized by high antibiotic tolerance.^{13,14,16} Persistence differs from antibiotic resistance in that upon regrowth of the persister cell fraction after initial antibiotic treatment, the bacteria retains sensitivity to the antibiotic and can consistently form the same persister cell fraction.¹⁷ Persister cells are strongly implicated in the pathogenesis of chronic infections, including recurrent UTIs where persistence of UPEC has been extensively studied.^{12,18-21} While proven to be different metabolically, quiescence and persistence both likely contribute to the ability of UPEC to cause recurrent UTI and treatment failures.¹² Therapies aimed at preventing or reversing quiescence and persistence could significantly reduce the burden of UPEC recurrent UTI.

Cranberry (*Vaccinium macrocarpon*) products have been extensively researched for their role in the treatment and prevention of UTIs especially in UPEC and other pathogens.²²⁻²⁷ While many studies have shown benefit, especially those completing *in vitro* experiments, the current clinical role of cranberry products in UTIs is controversial.²²⁻²⁶ Despite numerous completed studies, there is a lack of consistency in the type of study, study demographic, and especially the cranberry products and these inconsistencies can contribute to the conflicting evidence.²³ Specifically, current literature states that the major ‘active’ components are proanthocyanidins (PACs) with A-type linkages and fructose, both of which interfere with UPEC adherence to the bladder.^{23,25,28} However, in a comprehensive Cochrane review of 24 clinical studies, only five of the studies indicated a measurement of the amount of PACs in their tested products (juice, concentrate, capsules, or tablets), displaying the shortcomings of much of

the chemistry in the clinical trials completed thus far.²³ With inconsistent standardization of the cranberry chemistry, and inconsistent clinical evidence for its use in prevention of UTIs, we sought to determine if there were other therapeutic targets that cranberry chemistry could exploit to aid in the prevention of UTIs and recurrent UTIs, especially in UPEC. With the hypothesized importance of IBCs and QIRs to the infection cycle and recurrence of UTIs, as well as published studies detailing natural product compounds that are able to sensitize persister cells to antibiotics,²⁹⁻³¹ we hypothesized that compounds from cranberry may be able to reduce persistence and/or quiescence in UPEC. In the following study, we identify carbohydrate components from cranberry juice that are able to prevent quiescence in UPEC. Additionally, another subset of small molecules from cranberry juice significantly reduces the fraction of UPEC persister cells.

Results

Bioassay guided fractionation of active constituents from cranberry juice: A

pectinase (Klerzyme 150, DSM Food Specialties) degraded cranberry hull extract was purified using C18 flash chromatography to afford three fractions: the 100% water fraction, 15% MeOH fraction, and the 100% MeOH fraction. From previous work completed by our lab, it was known that the 15% MeOH fraction (Cranf1b) was enriched in oligosaccharides and was able to reduce biofilm formation in the UPEC CFT073.³² This fraction was further tested in our previously published CFT073 persister cell assay,¹² and showed a greater than 3-log reduction in persister cells at 24 h at 1 mg/mL (0.1% wt/vol) in the presence of ampicillin (0.1 mg/mL) when compared with ampicillin

treatment alone (**Figure 1**). Using CFT073 persister cell reduction to guide purification, we further purified Cranf1b using porous graphitized carbon (PGC) cartridges and determined that the second fraction (entitled 10% PGC) was similarly able to reduce the CFT073 persister cells more than 3 logs at a final concentration of 0.6 mg/mL (0.06%) (**Figure 2**). The 10% PGC fraction was further purified by semipreparative HPLC and analyzed using NMR and LC/MS to determine the chemical components of this fraction.

Structure elucidation of active constituents from cranberry juice: The ^1H and HSQC spectra of 10% PGC indicated that this fraction was enriched with oligosaccharides with methoxy group at 3.70 ppm and an additional correlation at 6.00/112.04 ppm in HSQC which together highly suggest the presence of a group of poly-galacturonic acid methyl esters with at least one hydroxyl group reduced. Therefore, this fraction was further fractionated using an amide column that provides superior separation of this class of molecules. The same amide column and program was used for LC-MS analysis and preparative purpose. Indeed, LC-MS chromatogram showed that the predominate ions presented were poly-galacturonic acid methyl esters of various degrees of polymerization. The most abundant ions were with one or two free carboxylic acids and with one degree of unsaturation. Surprisingly saturated poly-galacturonic acid methyl esters were minor ions in this fraction. Due to the ultra-high complexity of this fraction, only two unsaturated poly-galacturonic acid methyl esters were fully purified. The two pure unsaturated poly-galacturonic acid methyl esters were of 4-mer and 3-mer with one free carboxylic acid (compounds **4** and **5**, respectively). Due to the very limited quantity, only **4** was fully characterized with NMR and tested in the biological assays. The ^1H and ^{13}C NMR chemical shifts of **4** were listed in **Table S1**. Five spin systems were found in

total and were registered to the four subunits (A, B, C, D, **Figure 3**) in the oligomer. Key HMBC and NOESY correlations across glycosidic bond were shown in **Figure 3**. In addition, HMBC correlation between carbonyl carbon on each unit and methyl group was only found for subunit A, B and D, suggesting the free carboxylic acid was located on subunit C. The ESI-TOF-MS/MS fragmentation with sequential neutral losses of 190 Da in **4** was deduced as shown in (**Figure 4**) The dehydration at the 4- position on the galacturonic acid opposite of the reducing end was very much likely due to the enzymatic degradation during cranberry juice manufacturing to break down the insoluble pectic polysaccharides. Unlike acid hydrolysis, some pectinase can selectively hydrolyze the glycosidic bonds via eliminative cleavage and cause oxygen-aglycone bond breakage. This eliminative cleavage generally occurs by the free carboxylate group because of its strong electron withdrawing effect.³³⁻³⁵ Along with these unsaturated oligosaccharides, three previously reported iridoid glucosides were also isolated and identified to be 6,7-dihydromonotropein (**1**), deacetylasperulosidic acid (**2**) and monotropein (**3**) (**Figure S2**).³⁶⁻³⁹ To the knowledge of the authors, this is the first identification of **2** from *V. macrocarpon*.

Purified cranberry juice constituents and CFT073 quiescence: Using the compounds purified from cranberry juice, we investigated whether or not the inhibition on CFT073 persister cells could also be demonstrated with quiescent CFT073. 100 µg spots of each **4**, the 10% PGC fraction, mixed methyl galacturonic acid oligosaccharides fraction (Am-F6), and **1-3** were tested in the previously published quiescence lawn overlay assay.¹² The spot from the **4**, the 10% PGC fraction, and the mixed methyl galacturonic acid oligosaccharides fraction (Am-F6) all showed regrowth, thus demonstrating inhibition of

quiescence (**Figure 5**). It was notable that the growth with the pure **4** was not as dense as with the other active samples. Each of the tested iridoids (**1-3**) did not show an inhibition of quiescence.

Discussion

Despite years of practice, clinical trials, and bench research, the narrative surrounding the benefits of cranberry juice in UTIs is not fully understood. There are many explanations attempting to justify its use—from the increased acidity of the juice to the anti-adhesive compounds such as PACs—but clinical evidence fails to fully support the scientifically-derived benefits.^{3,23} The under-studied oligosaccharide components of cranberry juice discussed in this manuscript comprise a previously undiscovered benefit, though we have seen anti-biofilm effects with cranberry oligosaccharides previously.³² Pectic oligosaccharides formed in the presence of a juice-making pectinase are able to reduce persistence in UPEC. Over a 1000-fold decrease in persistent UPEC could have a tremendous effect on treatment outcomes as there would be less cells ‘avoiding’ the antibiotic treatment through persistence. It is important to consider that any positive effects of cranberry oligosaccharides on persister cells could only be appreciated in the presence of antibiotics, so this effect could be defined as a ‘treatment’ benefit. However, the inhibition of quiescence in UPEC seen with the cranberry oligosaccharides could directly hinder the ability of the UPEC to establish a QIR, thereby reducing the likelihood of a recurrent UTI.⁶ Inhibition of quiescence does not require antibiotics and could therefore be loosely interpreted as a ‘preventative’ effect on recurrent UTIs. While the *in*

vitro effects described in this manuscript are distinct from one another, it is important to remember that persistence and quiescence are similar biological states. It is beyond the scope of this manuscript to determine if the physiologic change in UPEC associated with cranberry oligosaccharides is consistent between the anti-persistence and anti-quiescent effects. Investigations into the biological manifestations of reducing persistence and quiescence in UPEC are necessary next experiments to further understand the beneficial role of these oligosaccharides.

While the completed experiments are preliminary, the results strongly suggest that cranberry oligosaccharides formed during the juice-making process have the potential to benefit sufferers of UTIs caused by *E. coli* and especially in those suffering from recurrent *E. coli* UTIs. It is rational to surmise that the full benefit of cranberry juice may not be realized with one class of chemical compounds, but multiple. From our previous chemical purification of oligosaccharides, we know that enrichment of the oligosaccharides is nearly synonymous with removal of phenolic compounds, including the important type A PACs.³² One could assume that a phenolic-enriched (or PACs enriched) fraction might be similarly depleted of oligosaccharides, including those identified in this study. If the ultimate goal is to understand what the full benefit of cranberry juice components are in UTIs, more careful chemistry is needed to assure that the preparations tested include the right compounds, in sufficient quantity. Exhaustive reviews of the clinical data show clearly that inconsistencies in the chemical standardization are rampant in the literature.²³ Using the juice as the supplement is the obvious answer to including all the components, but there would still need to be clarity

on how much of the juice is necessary to drink, especially in the case of the oligosaccharide content.

There is still much work left to do to determine if cranberry oligosaccharides can have a meaningful effect on the treatment or prevention of UPEC UTIs.

Pharmacokinetic, *in vivo* modeling, and mechanistic experiments are necessary to elucidate the answer. While these gaps in the research are significant, the current data invites a new class of compounds to be considered as part of the ‘active’ component of cranberry juice. We strongly feel that chemical preparations of cranberry meant to yield benefits in UTIs should not exclude oligosaccharides or they may be missing a crucial component to cranberry’s anti-infective cocktail.

Methods

General experimental procedures: NMR experiments were conducted using an Agilent NMRS 500 MHz spectrometer in D₂O (99.99%, Sigma-Aldrich) at 25 °C. LC/MS analysis was performed on a Shimadzu Prominence UFLC system coupled to an AB Sciex Qtrap 4500 mass spectrometer with electrospray ionization source in the negative ion mode. High Resolution Electrospray ionization mass spectra (HR-MS) were acquired using an AB Sciex TripleTOF 4600 spectrometer in the negative ion mode. Flash chromatography was completed with a Combiflash Rf200 equipped with a 100 g C18 RediSepRf High Performance Gold column (Teledyne ISCO). HPLC purification experiments were performed on a Shimadzu Prominence i-series HPLC.

Purification of cranberry compounds: Cranberry hulls were degraded with pectinase (Klerzyme 150, DSM Food Specialties) and fractionated as previously described with modifications.³² Briefly, 2 g of the cranberry pectinase treated powder was dissolved in 20 ml distilled water and purified using flash chromatography. The column was eluted sequentially with 500 ml DI water, 500 ml 15% methanol/water and 500 ml methanol. Fractions from each gradient were individually pooled and lyophilized to obtain three major fractions, Cranf1W (761 mg, 38.1%) eluted with 100% DI water, Cranf1b (476 mg, 23.8%) eluted with 15% methanol/water, and Cranf1M (562 mg, 28.1%) eluted with 100% methanol. The Cranf1b was then subject to purification using a porous graphitized carbon (PGC) cartridge (1 g, Thermofisher Scientific). The PGC cartridge was first conditioned by eluting with 50% ACN/water and then equilibrated with DI water. For each round of PGC purification, 40 mg of Cranf1b was dissolved in 2 mL of DI water, loaded onto the PGC cartridge, and eluted sequentially with 18 mL DI water, 12 mL 10% ACN/water (+0.1% TFA), and 30% ACN/water (+0.1% TFA). Fractions were individually pooled and dried *in vacuo* to obtain three major fractions: 100% water PGC, 10% PGC, and 30% PGC. The 10% PGC fraction was then purified using HPLC and a TOSOH Tskgel Amide-80 HR (4.6 × 250 mm, 5 µm) (TOSOH Bioscience) at 1 mL/min and 35°C. The column was initially eluted with 80% ACN/water (+0.1% formic acid) for 2 min, followed by a linear gradient to 40% ACN/water (+0.1% formic acid) over 30 min and held for 5 min. This afforded six fractions (**Figure 3**): Am-F1 (2.5-8 min), Am-F2 (peak at 8.6 min, mixture of **1** and **2**), Am-F3 (9 min, **3**), Am-F4 (split peak at 12 min), Am-F5 (split peak at 14 min, **4**), and Am-F6 (15-25 min). The wavelength of UV absorbance was set at 235 nm for optimal monitoring of dehydro-poly-galacturonic acids.

Am-F2 was further purified by semipreparative HPLC with a Waters Xbridge C18 column (4.6×250 mm, 5 μ m) at 1 mL/min 35°C with isocratic 5% MeOH/water (+0.1% Formic acid) for 20 min to afford pure **1** and **2** (**Figure 4**).

Structure elucidation of purified compounds: The LC/MS analysis was performed on the same Amide-80 column and same HPLC program as in “Purification of cranberry compounds.” The mass spectrometer was operated in negative mode and ions from m/z 200 to m/z 2000 were scanned. Compounds **1-4** were subject to one and two dimensional NMR experiments to determine structures. Additionally, molecular weights were determined for pure compounds using HRMS. For necessary MS/MS experiments, collision energy was set to 70.

Bacterial strains and cultivation: *E. coli* CFT073 was obtained from a cryo stock at the lab of P.S.C. and stored at -80°C in a 1:1 mixture of LB broth and 50% glycerol by volume. LB broth and LB agar were used for routine cultivation. Liquid M9 minimal media was prepared as before,¹² and M9 minimal media agar plates were prepared with 1.5% noble agar to avoid impurities present in bacteriological agar.

Persister cell viability assay: The procedure for this assay closely follows a published protocol.¹² In general, CFT073 was streaked from cryo stocks onto LB agar plates and incubated overnight at 37°C. A loopful of cells from the plate was added to 10 mL 0.4% glucose M9 minimal media in a 125 mL culture flask and incubated overnight at 37°C and 200 rpm. Cultures were diluted in fresh 0.2% glucose M9 minimal media to an optical density (OD₆₀₀) of 0.1 ($\sim 10^8$ CFU/mL) and grown in the presence of ampicillin sodium (0.1 mg/mL) to generate persister fractions. The cultures were incubated shaking

(200 rpm) at 37°C and viable counts were measured at 0, 4, and 24 hours by plating on LB media.

Quiescence lawn assay: The procedure for this assay follows closely follows a published protocol.¹² In general, overnight cultures of CFT073 were prepared in 0.4% glucose M9 minimal media as described in “Persister cell viability assay.” Bacteria from this culture were diluted to a final concentration of 10^5 CFU in 4 mL of liquid overlay media (0.2% glucose M9 minimal media with 0.9% noble agar at 45°C). Each 4 mL overlay inoculum was poured over a prewarmed (37°C) 0.2% glucose M9 minimal media agar plate immediately after inoculation. These plates were allowed to solidify at room temperature with lids slightly ajar. Test solutions or bacteria were added to the overlay media and allowed to dry before incubating the plate upside down at 37°C for 24 or 48 hours as indicated. No growth of *E. coli* in the overlay was considered to be quiescence if it was able to be reversed with the positive control (3 co-spots of 5 μ L of each tyrosine, lysine, and methionine at 0.1 mg/mL).

Statistics: Persister assays were completed in triplicate and compared using a two-tailed student's *t* test. *P*-values < 0.05 were considered statistically significant.

Acknowledgements: This work was supported, in part, by Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA, USA). RD was financially supported by the Omar Magnate Foundation Fellowship. Research reported in this publication was made possible by the use of equipment and services available through the RI-INBRE Centralized Research Core Facility, which is supported by the Institutional Development Award (IDeA)

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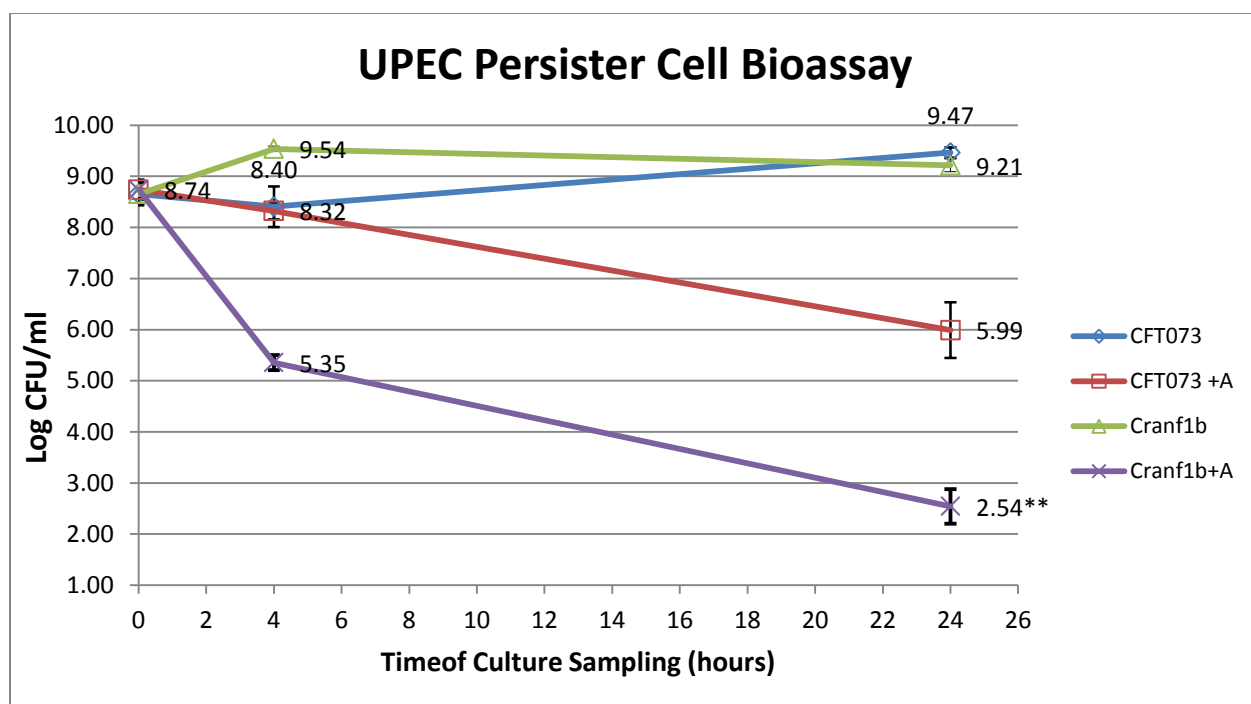


Figure 1: Persister cell viability assay using Cranf1b fraction as the test sample. **-

statistically significant, A- ampicillin.

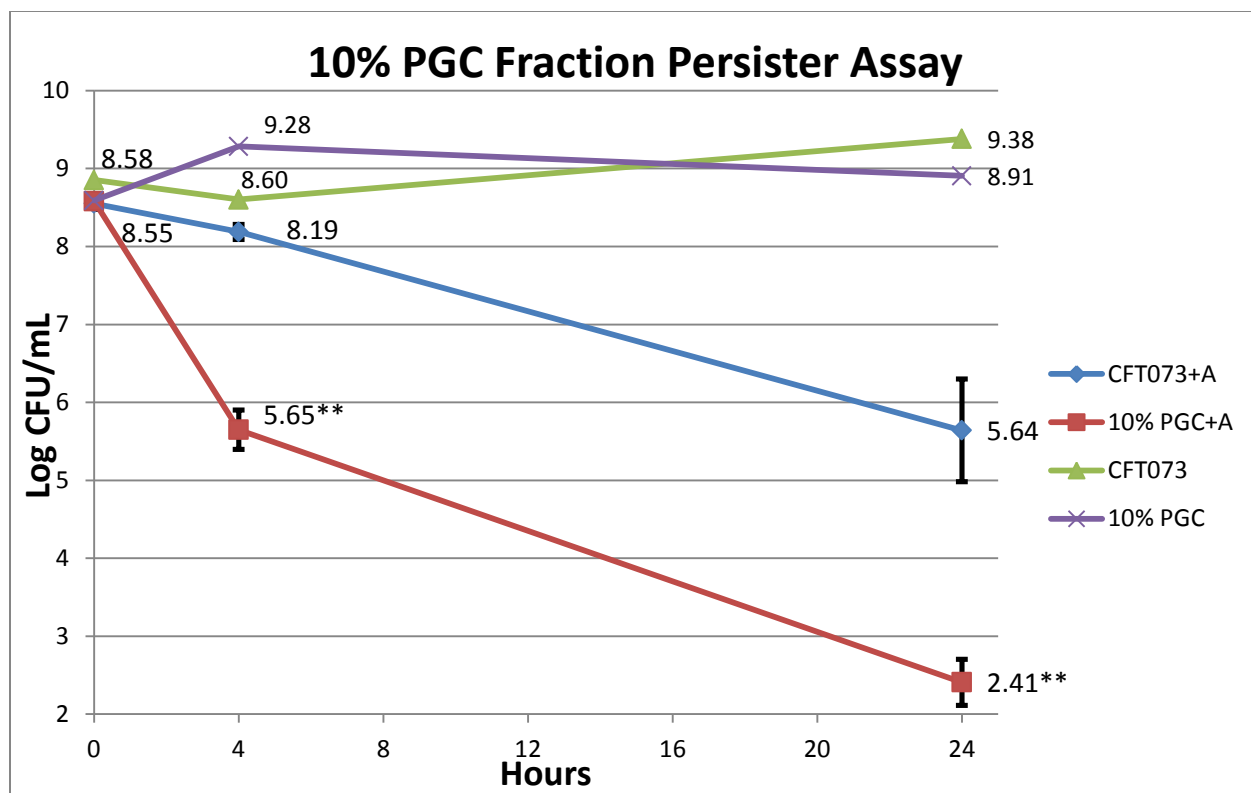


Figure 2: Persister cell viability assay using 10% PGC fraction as test sample. **- statistically significant, A- ampicillin.

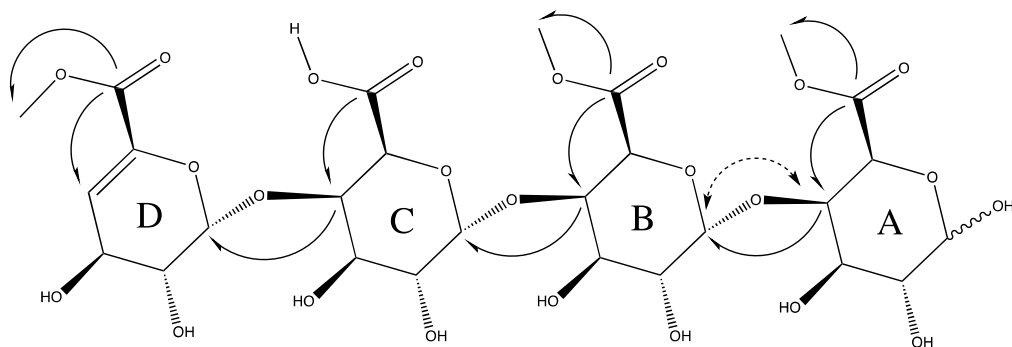


Figure 3: Subunits of **4**. Key HMBC correlations are designated with single-headed arrows and key NOESY correlations are designated by double-headed, dotted arrows.

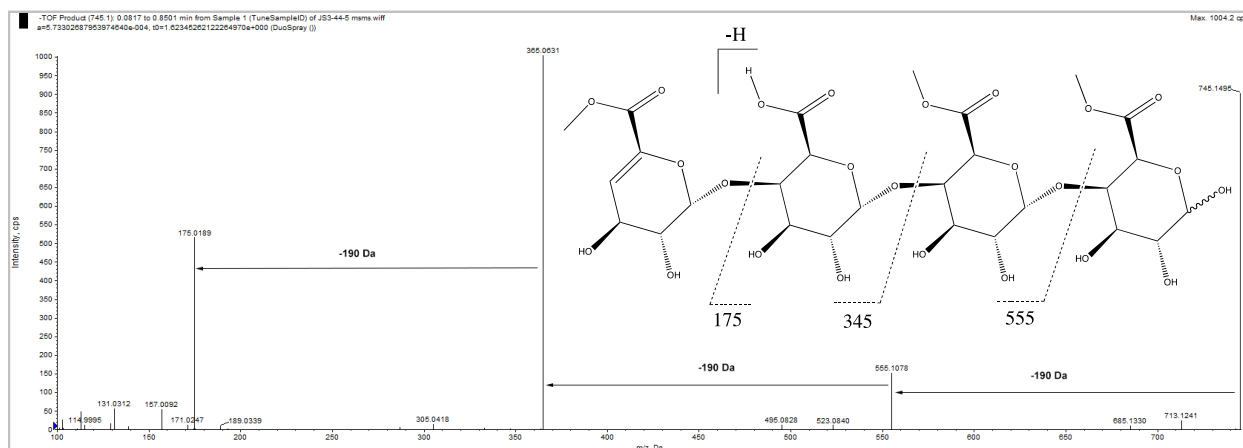


Figure 4: ESI-TOF MS-MS of **4**. Structure fragments are designated with the corresponding fragment ions.

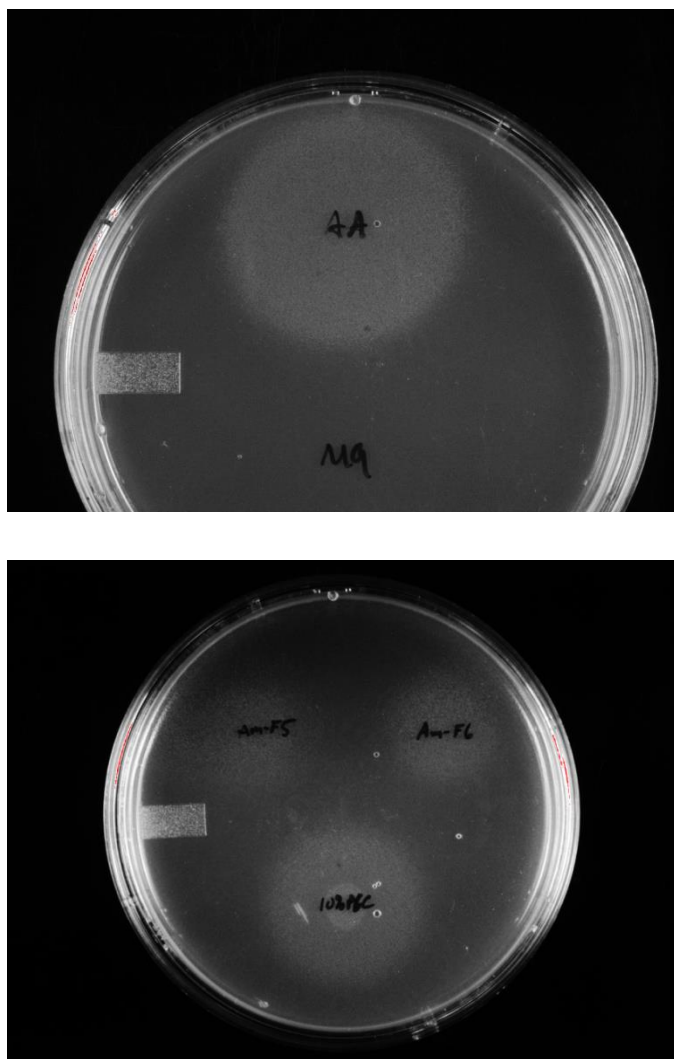


Figure 5: Representative quiescence overlay assay plates. Upper image: AA- amino acid mixture, positive control; M9- negative control. Lower image: Am-F5- **4**; Am-F6- mixture of methylated galacturonic acid oligosaccharides; 10% PGC- fraction described in text.

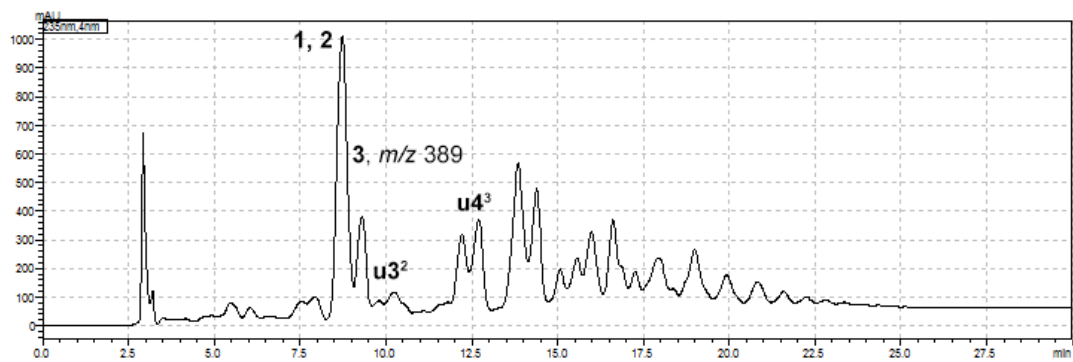


Figure 6: Amide-80 HPLC chromatogram of 10% PGC.

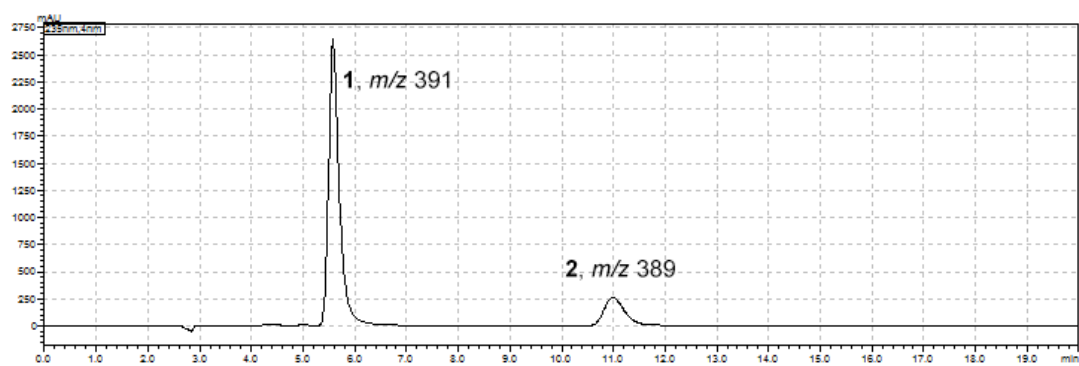


Figure 7: C18 Reverse-phase HPLC chromatogram of **1** and **2** mixture.

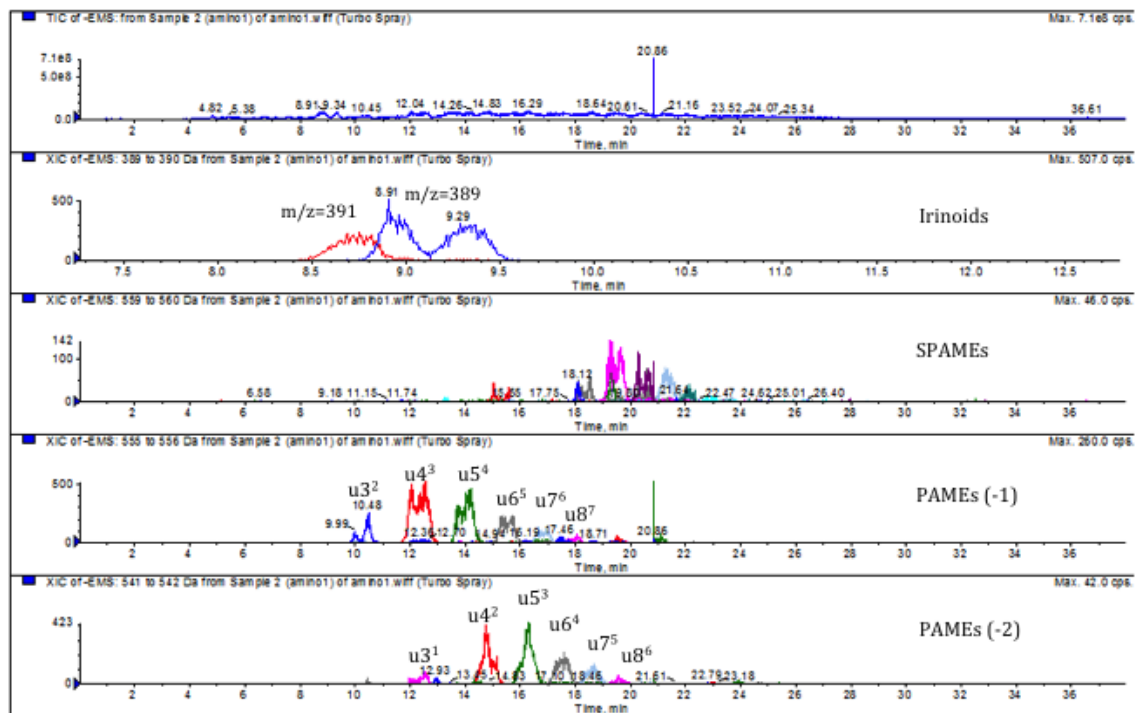


Figure 8: LC-MS analysis of 10% PGC. From top to bottom: Total ion chromatogram; Extract ions of m/z 391 and m/z 389; Extract ions for saturated poly-galacturonic acid methyl esters (DP 3-8); Extract ions for unsaturated poly-galacturonic acid methyl esters (DP 3-8), single free carboxylic acid; Extract ions for unsaturated poly-galacturonic acid methyl esters (DP 3-8), double free carboxylic acids.

CHAPTER 6

Perspective

The current paradigm for treating bacterial infections is about identifying the causative pathogen, and choosing an antibiotic that is capable of killing or inhibiting the growth of that particular pathogen. This is the perfect system for developing antimicrobial resistance because these antibiotics that kill or inhibit growth are the exact types of environmental stressors that a bacterium would need to develop a mechanism for circumventing in order to survive. As such, bacterial resistance to antibiotics is growing at an alarming rate and actions are urgently needed to avert this impending pandemic.

The identification of new antibacterial molecules and the development of these chemical compounds into medicines remains a crucial component in our arms race against bacteria. Declining participation in antibiotic research from the pharmaceutical industry does not help this problem. With fewer resources and time at the disposal of researchers, we must choose wisely where we search for these new antibacterial agents that we desperately need. Investigating natural products (especially those from microorganisms) has been by far the most successful method for finding molecules that become antibiotic drugs, and this remains a viable resource going forward. Importantly, while natural products research is thought of as a ‘classical’ research method, it has been decades since the development of natural antibacterial compounds was commonplace in industry and there have been thousands of antibacterial compounds isolated and published since. In addition, with new frontiers such as microorganisms from the marine environment to explore, the microbial natural resource of antibacterial agents remains largely undiscovered.

Continuing to fight fire with fire- that is killing bacteria with antibiotics until they develop resistance to them- is a never-ending battle. In order to fully sustain the monumental achievement of antibiotics, we must target the very mechanisms of resistance and the characteristics of bacteria that make them pathogenic to find new ways to treat infections. Efflux pumps and the subsequent efflux pump inhibitors are one good example of a way to reclaim antibiotics that are losing their potency. Other molecules have shown utility as drugs to re-sensitize resistant pathogens to antibiotics. Additionally, targeting mechanisms of pathogenicity such as persistence and quiescence in this manuscript, or other characteristics like biofilms and quorum sensing are viable avenues towards stopping infections without stimulating resistance. Also beyond antibacterial compounds, we can learn new ways to treat infections from simple things like cranberry juice. Oligosaccharides with no antibacterial effect whatsoever through a different biological lens look particularly important to the juice's effects in UTIs. We must increase our understanding of how and why the pathogens are causing infections in order to find the best ways of treating them.

I will end this dissertation with a few broadly reaching statements and some thought provoking questions from working in this particular area of research:

Preventing an infection is far better than having to treat it, but bacteria are such a huge part of human life, it seems no amount of standardized precautions would stop infections from happening all together, that being said, we must have viable ways to treat them or we face losing vital aspects of medicine that are taken for granted. Currently, research into developing truly new ways to treat bacterial infections is a bare industrial landscape. Who is going to pay for clinical trials of non-antibiotic agents for infections if

the industry will not? Or better yet, what do we have to change in our health care system to incentivize developing antibiotics and similar agents? Perhaps we as a society are not yet ready to make these changes and commit to whatever it takes to preserve our antibacterial luxury. Or maybe the data is not compelling enough to force change in this developmental landscape. What will be the cost of failing to develop new antibiotics for 5 more years? What about 10 or 20? I shudder to think about pan-drug resistant *Acinetobacter baumannii* colonizing the majority of our hospitals. Do we really know how long it will take for something like that to happen? If we only lose 23,000 lives in this country to MDR bacteria per year now, how many lives must be lost per year for the general public to become aware of this issue? I sincerely hope that the problem of antimicrobial resistance will be solved quietly, but I fear that the noise must become louder to draw the attention necessary to prevent the impending post-antibiotic era.

APPENDIX A

A Marine Bacteria-Produced Benzisoxazole with Antibacterial Activity Against Multi-Drug Resistant *Acinetobacter baumannii*

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List of Supplementary Material

1. Additional Results:

- 1.1 Bioassay Screening of *B. denitrificans* B158 Extract
- 1.2 Biofilm Formation Assay for Characterization of *A. baumannii* Strains
- 1.3 Isolation and dereplication of additional compounds from *B. denitrificans* B158

2. Supplementary References

- 3. **Table S1:** ¹H and ¹³C NMR data of **12** and comparison to published data
- 4. **Table S2:** *A. baumannii* strain characteristics and resistance profiles
- 5. **Figure S1:** Chemical structures of compounds **11** and **12**
- 6. **Figure S2:** LC/MS comparison of isolated **1** to synthetic **1**
- 7. **Figure S3:** ¹H NMR of isolated **1**
- 8. **Figure S4:** ¹H NMR of synthesized **1**
- 9. **Figure S5:** ¹³C NMR of synthesized **1**

Additional Results

1.1 Bioassay Screening of B. denitrificans Extract

Extract from isolate B158 reduced the MIC of erythromycin >4-fold when tested against *E. coli* MDR strains MG1655 Δ BC/pABM, and MG1655 Δ BC/pXYM according to previously published methods.[1] These strains overexpress two Resistance Nodulation cell Division (RND) transporters involved in antibiotic resistance. Bioassay-guided fractionation of the B158 extract resulted in the isolation of **1** with an MIC between 0.63 – 1.25 μ g/mL against *E. coli* MDR strains MG1655 Δ BC/pABM, and MG1655 Δ BC/pXYM.

1.2 Biofilm Formation Assay for Characterization of A. baumannii Strains

Quantification of biofilm formation was conducted using a modified microtiter plate assay [2-4] previously described by Song et al.[5] Briefly, strains were grown overnight on Tryptic Soy Agar (TSA, Becton–Dickinson, Sparks, MD, USA). Tryptic Soy Broth (TSB; Becton–Dickinson, Sparks, MD, USA) supplemented with 1% dextrose (for total dextrose concentration of 1.25%) was used to optimize biofilm production in the biofilm assay. A 0.5 McFarland standard of overnight growth of test isolates was diluted into TSB. The inoculated medium was dispensed into wells of sterile non-tissue culture treated flat-bottom 96-well polystyrene plates (Costar no. 3370; Corning Inc., Corning, NY, USA). Plates were incubated on a shaker at 37 °C. After 24 h of biofilm development, broth was removed and replaced with fresh TSB and incubated at 37 °C for an additional 24 h. The solution was then removed and plates were carefully rinsed three times with sterile water to remove planktonic bacteria. Adherent bacteria were dried overnight and stained for 15 min with 0.1% crystal violet solution. The crystal violet was

then resolubilized in 33% glacial acetic acid and the optical density (OD) of stained adherent bacterial films was read at 570 nm using a spectrophotometer (ELX800, Biotek, Winooski, VT). The OD readings of bacterial films were categorized into non-adherent, weak, moderate, and strong based on previously described methods by Stepanovic et al.[6] Isolate L1051 was used as a negative control strain. Experiments were carried out in quadruplicate. OD readings were averaged, and standard deviations were calculated for each isolate.[2,4]

1.3 *Isolation and Dereplication of Additional Compounds from B. denitrificans B158*

Briefly, 1152.4 mg of extract was applied to a silica gel column (40-60 μm , 60 Å) and eluted with a step-gradient of isooctane, (4:1) isooctane/ethylacetate (EtOAc), (3:2) isooctane/EtOAc, (2:3) isooctane/EtOAc, (1:4) isooctane/EtOAc, EtOAc, (1:1) EtOAc/MeOH, and 100% MeOH, yielding eight fractions. Bacterial susceptibility testing in the INT assay indicated activity to be localized to the EtOAc elution. The active fraction totaling was separated further into 96-deep well plates by semipreparative HPLC using an Agilent 1200 series HPLC and a Phenomenex Luna® 5 μm C₁₈ (2) 100 Å, LC column (250 x 10 mm) as the stationary phase, heated to 30 °C and a gradient of water and MeCN with a flow rate of 4 mL/min. All solvents were acidified with 0.1% FA. Contents of the 96-deep well plate were dried down and tested in the INT assay, which resulted in identifying three wells (E7, F7, and G7) eluting at 35:65 water/MeCN were active. Contents of wells E7, F7, and G7 were combined and separated further into a 96-deep well plate by semipreparative HPLC using an Agilent 1200 series HPLC and a Phenomenex Luna® 5 μm C₁₈ (2) 100 Å, LC column (250 x 10 mm) as the stationary phase, heated to 30 °C and a gradient of water and MeCN with a flow rate of 4 mL/min.

All solvents were acidified with 0.1% FA. The activity was found to be contained in a single well (H10) eluting at 22.8 min corresponding to 5:95 water/MeCN visualized by a single sharp peak at 276 nm. The contents of well H10 underwent an additional round of HPLC purification using the above HPLC column, flow rate, but with starting conditions of 1:1 water/MeCN. INT activity testing indicated a well resolved peak with minor activity eluting at 4.5 min in 1:1 water/MeCN to contain all of the activity and totaling 2.6 mg of pure **11** as an amorphous yellow powder.

Structure elucidation efforts of **11** were pursued. An $[M+H]^+$ ion of 243.0879 using HRESIMS indicated a molecular formula of $C_{12}H_{10}N_4O_2$. Examination of the 1H NMR displayed two singlet methyl groups (δ_H 2.46, 3H, and δ_H 2.49, 3H) and two singlet aromatic protons (δ_H 7.70, 1H and δ_H 7.91, 1H). These molecular features along with the molecular formula strongly suggested the known microbial metabolite, 7,8-dimethylalloxazine or lumichrome. This was further confirmed by NMR comparison to literature [7] and LC/MS comparison (data not shown) to an authentic standard of lumichrome (Cat. 103217, Sigma-Aldrich). A total of 2.6 mg of compound **11** was isolated from 1152.4 mg of extract from 9 L of microbial culture.

Compound **12** was purified from the 96-deep well plate fractions H8, A9, B9, totaling 25.1 mg, and had minor activity when tested in the INT assay. The 25.1 mg fraction containing wells H8, A9, B9 was further separated by semipreparative HPLC (Shimadzu Prominence-i LC-2030C). Compound **12** eluted at 15.7 min from the following method: 0 to 4 min, 30% MeOH in aqueous 0.1% FA; 4 to 24 min, gradient from 30% MeOH in aqueous 0.1% FA to 70% MeOH in aqueous 0.1% FA. This afforded **12** as a white amorphous powder. The molecular formula was determined to be

$C_{10}H_8N_2O_2$ using HRESIMS (m/z 187.0531 $[M-H]^-$) and NMR. 1H -NMR indicated the presence of a 1,2 substituted benzene ring based on resonances at δ_H 7.25 (m, 2H, H-5 and H-6), 7.53 (dd, 1H, H-7, $J = 5.7, 2.2$), and 8.22 (dd, 1H, H-4, $J = 5.6, 2.5$). In addition, a singlet at δ_H 8.68 (1H, H-2) and MS/MS fragmentation experiments showing a strong fragment at m/z 116 indicated that the aromatic protons were part of an indole subunit (data not included). Using our molecular formula and the indole substructure, **12** was dereplicated as (1*H*-indol-3-yl) oxoacetamide by comparison of 1H and ^{13}C -NMR to published data (**Table S1**) [8]. A total of 25.1 mg of **12** was isolated from 18.2 g of extract representing 118.5 L of microbial culture. Testing in bacterial susceptibility assays indicates **12** has antibiotic-like properties against *E. coli* MDR strains MG1655 $\Delta BC/pABM$, and MG1655 $\Delta BC/pXYM$ resulting in a reduction of 50% growth at 150 $\mu g/mL$.

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Table S1: Comparison of ^1H and ^{13}C -NMR data of **12** and published (1*H*-indol-3-yl) oxoacetamide. Chemical shifts are referenced to residual DMSO in $\text{DMSO}-d_6$ at δ_{H} 2.50 and δ_{C} 39.5.

	Isolated (1 <i>H</i> -Indol-3-yl) oxoacetamide (12)		Published (1 <i>H</i> -Indol-3-yl) oxoacetamide[1]	
position	δ_{C} , type	δ_{H} , (<i>J</i> in Hz)	δ_{C} , type	δ_{H} , (<i>J</i> in Hz)
1		12.35, br s		12.19, br s
2	138.2, CH	8.68, s	138.1, CH	8.69, s
3	112.0, C		112.0, C	
3a	126.1, C		126.1, C	
4	121.2, CH	8.22, dd (5.6, 2.5)	121.2, CH	8.22, d (6.0)
5	122.4, CH	7.25, m	122.4, CH	7.25, t (6.0)
6	123.3, CH	7.25, m	123.3, CH	7.25, t (6.0)
7	112.5, CH	7.53, dd (5.7, 2.2)	112.4, CH	7.52, d (6.0)
7a	136.3, C		136.2, C	
8	182.9, C		182.9, C	
9	166.0, C		165.9, C	
NH ₂		8.05 br s		8.06 br s
		7.69 br s		7.69 br s

Table S2: *A. baumannii* strain characteristics and resistance profiles.

<i>A. baumannii</i> strain	Source	Biofilm OD₅₇₀ readings	Biofilm Adherence**
L1051*	Clinical	0.19± 0.03	0
L1222*	Blood	0.60± 0.12	1
L1227	Blood	0.94± 0.27	2
L1184	Urine	2.49± 0.17	3

* Multi-drug resistant organisms (MDRO)

**0-non-adherent, 1-weakly adherent, 2- moderately adherent, 3- strongly adherent

Figure S1: Chemical structures of compounds **11** and **12**.

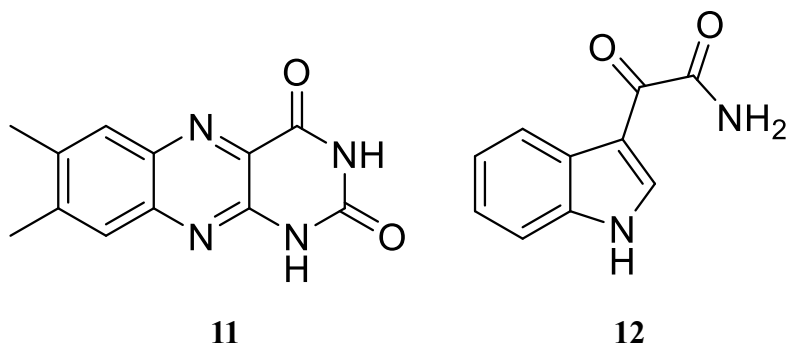


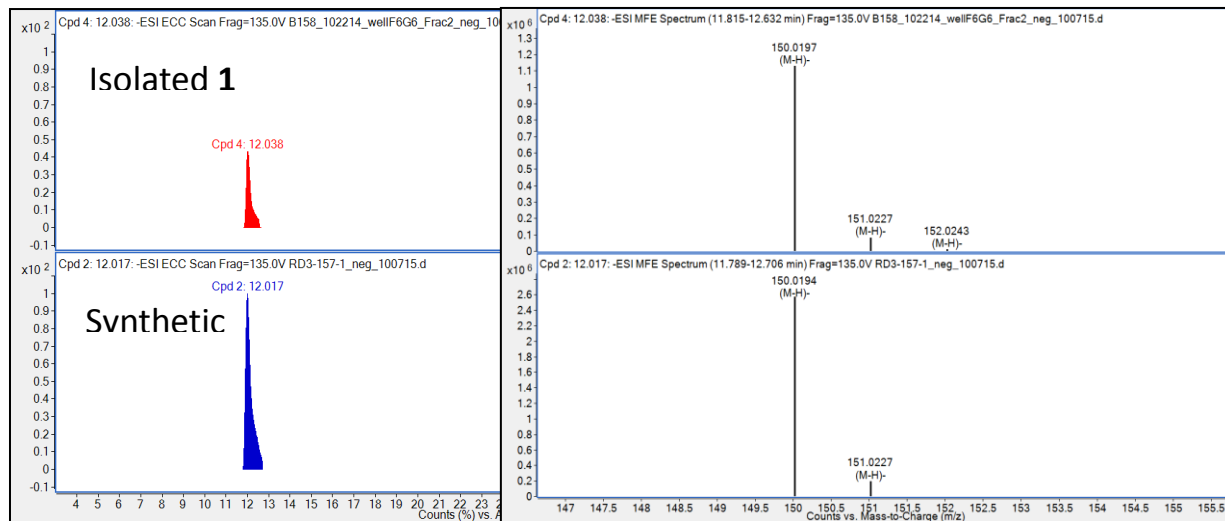
Figure S2: LC/MS comparison of isolated **1** to synthetic **1**.

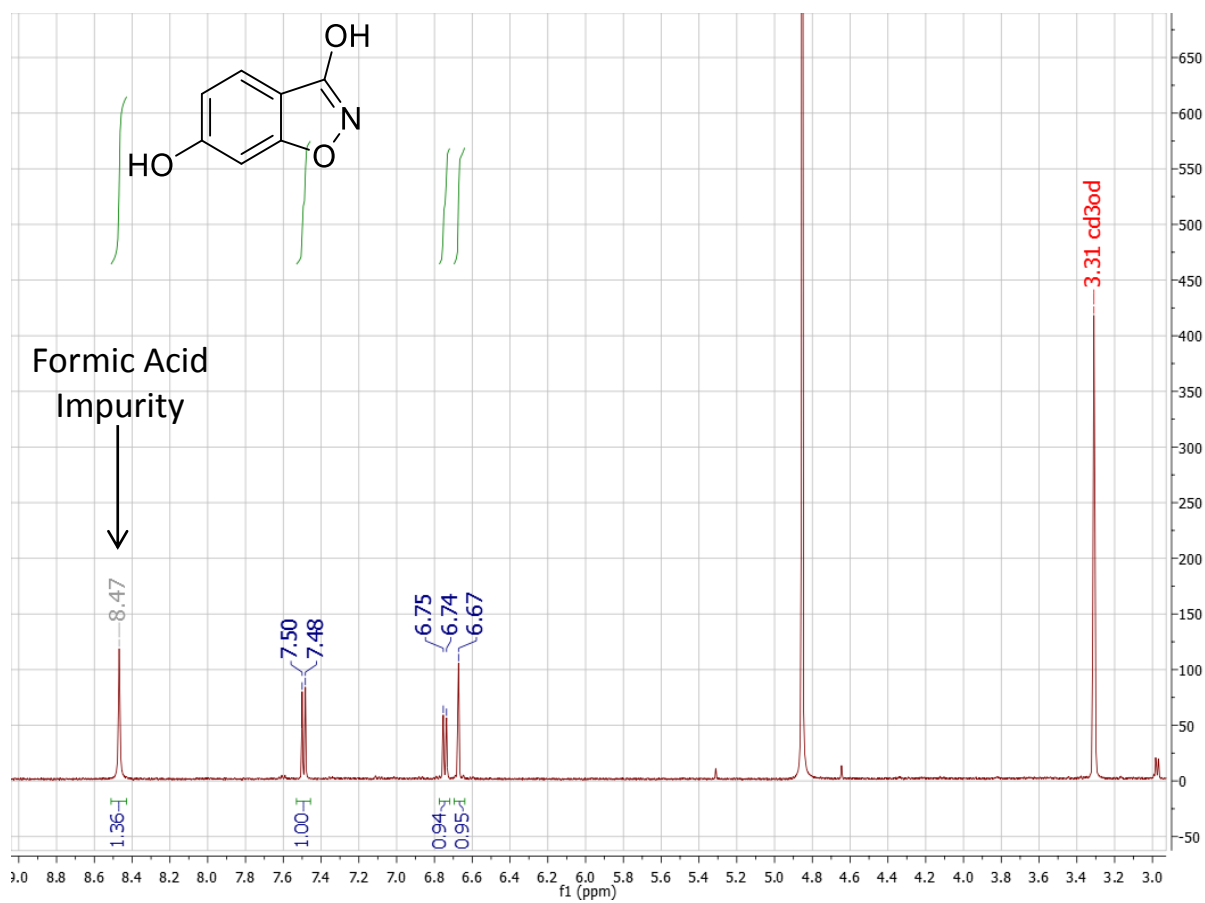
Figure S3: ^1H NMR of isolated **1**

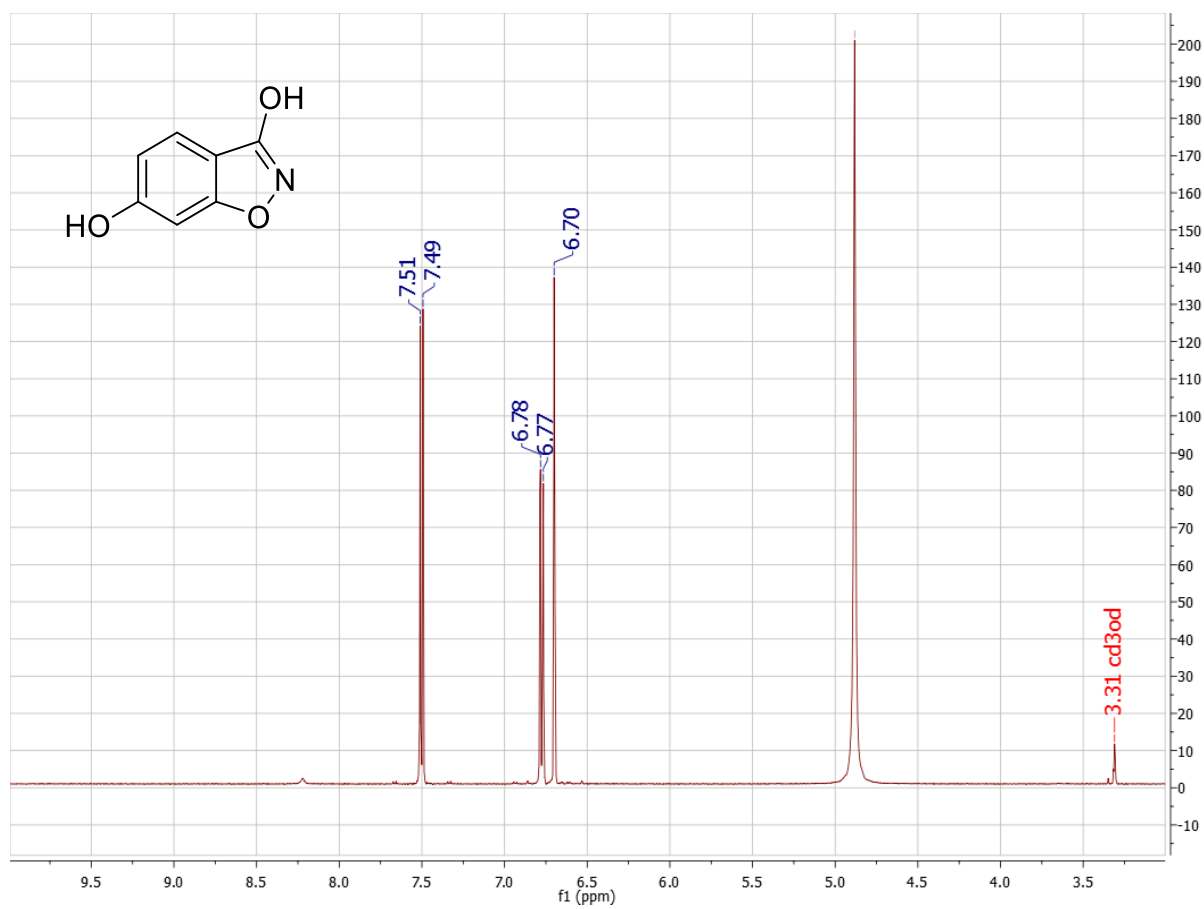
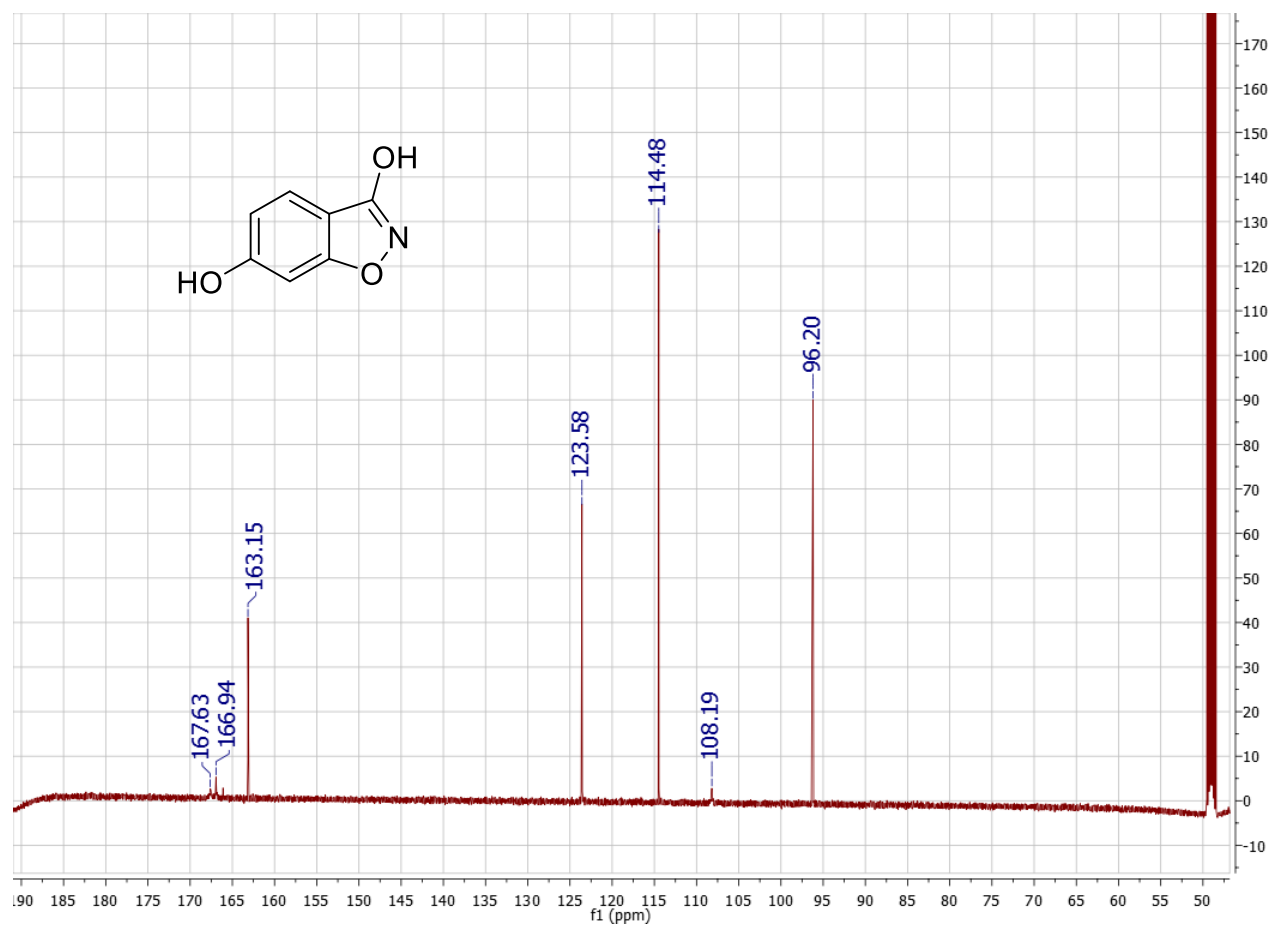
Figure S4: ^1H NMR of synthesized **1**

Figure S5: ^{13}C NMR of synthesized **1**

APPENDIX B

Supplemental Figures for Chapter 5

	A (α -)		A (β -)		B		C		D	
Position	δ C (ppm)	δ H (ppm)	δ C (ppm)	δ H (ppm)	δ C (ppm)	δ H (ppm)	δ C (ppm)	δ H (ppm)	δ C (ppm)	δ H (ppm)
1	92.3	5.25	96.3	4.55	100.5	4.88	100.0	4.80	99.5	5.04
2	67.7	3.71	71.2	3.38	67.8	3.62	68.0	3.57	69.8	3.65
3	68.0	3.93	67.8	3.69	67.8	3.92	68.4	3.83	65.5	4.23
4	78.8	4.36	78.5	4.31	78.2	4.39	79.0	4.43	111.8	6.00
5	69.7	4.72	73.1	4.40	70.4	4.99	71.2	4.64	140.6	
6	170.5		169.6		170.7		175.0		164.0	
7	52.8	3.70	52.8	3.70	52.8	3.70			52.8	3.71

Table S1: ^{13}C and ^1H NMR chemical shifts of **4**.

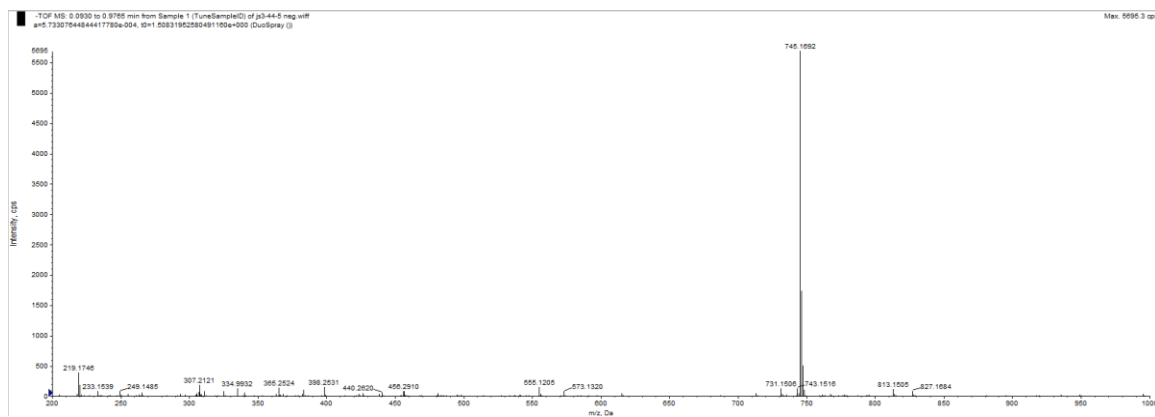


Figure S1: High resolution ESI-TOF-MS of **4**.

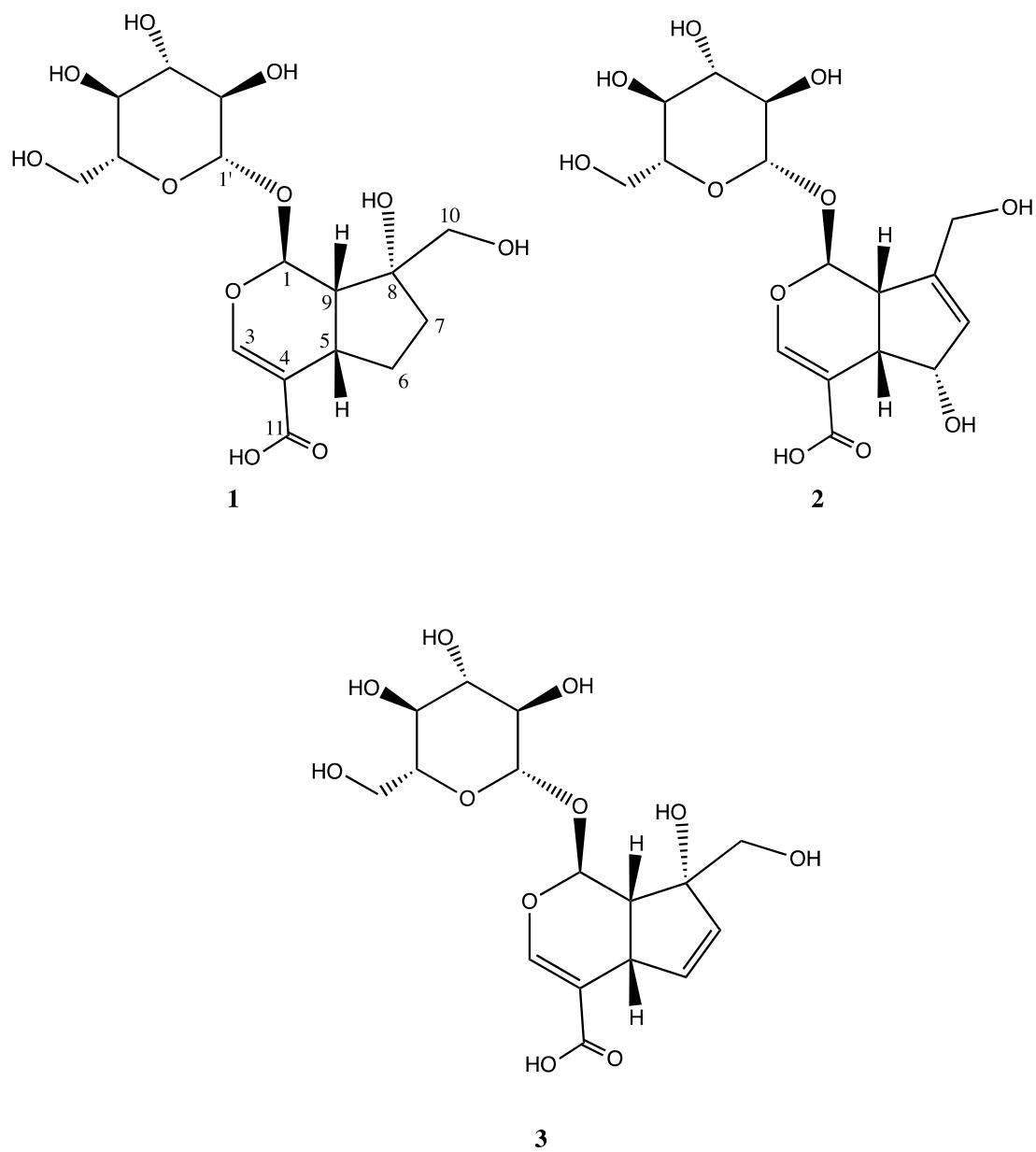


Figure S2: Structures of Iridoid glycosides **1-3**.

1			2		3	
Position	δ C (ppm)	δ H (ppm)	δ C (ppm)	δ H (ppm)	δ C (ppm)	δ H (ppm)
1	94.8	5.42	97.3	5.04	93.7	5.44
3	152.0	7.40	148.3	7.08	144.4	7.47
4	112.0					
5	32.0	2.84	44.8	2.89	38.3	3.43
6	29.4	1.93/1.57	80.8	4.41	137.9	6.10
7	34.9	1.70/1.56	128.5	5.75	137.2	5.56
8	81.8					
9	45.4	2.24	45.9	2.94	44.1	2.56
10	67.6	3.47	59.8	4.22/4.17	66.6	3.54
11	191.3					
1'	98.5	4.71	98.2	4.68	97.9	4.67
2'	72.5	3.17	72.6	3.22	72.8	3.18
3'	75.6	3.32	75.6	3.40	75.7	3.37
4'	69.4	3.31	69.1	3.31	69.5	3.29
5'	75.8	3.38	76.1	3.30	75.9	3.30
6'	60.5	3.80/3.62	60.4	3.78/3.60	60.6	3.80/3.62

Table S2. ^{13}C and ^1H NMR chemical shifts of **1**, **2** and **3**.

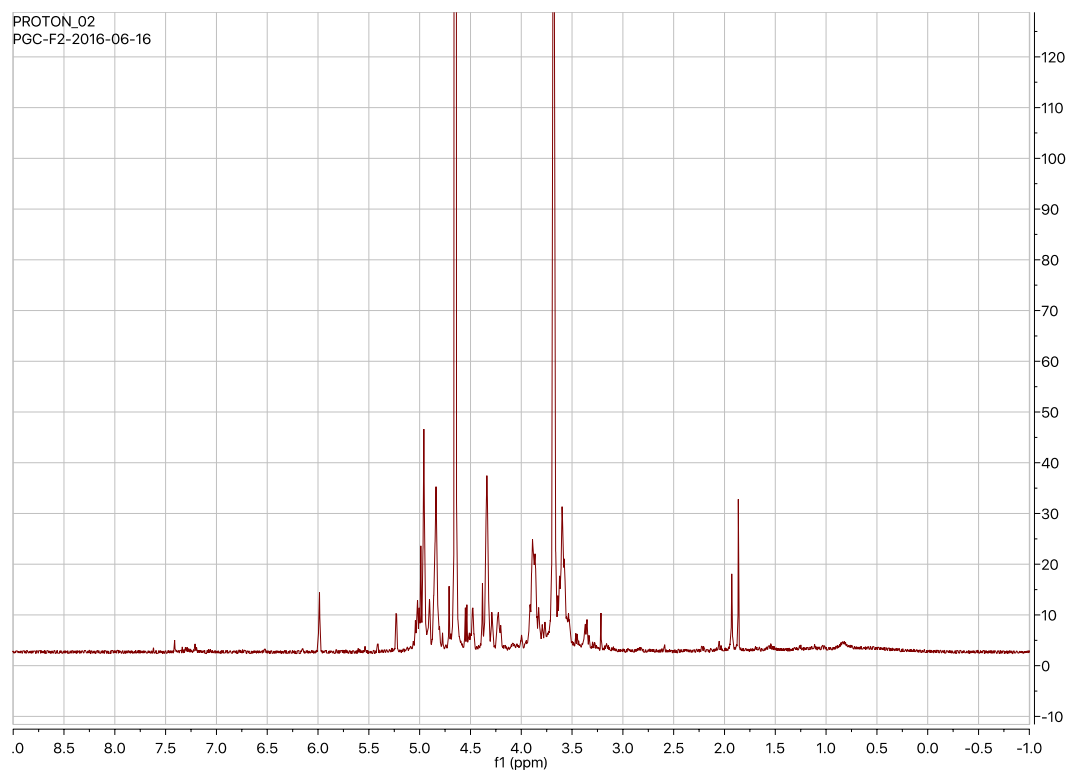


Figure S3: ^1H NMR spectrum of 10% PGC.

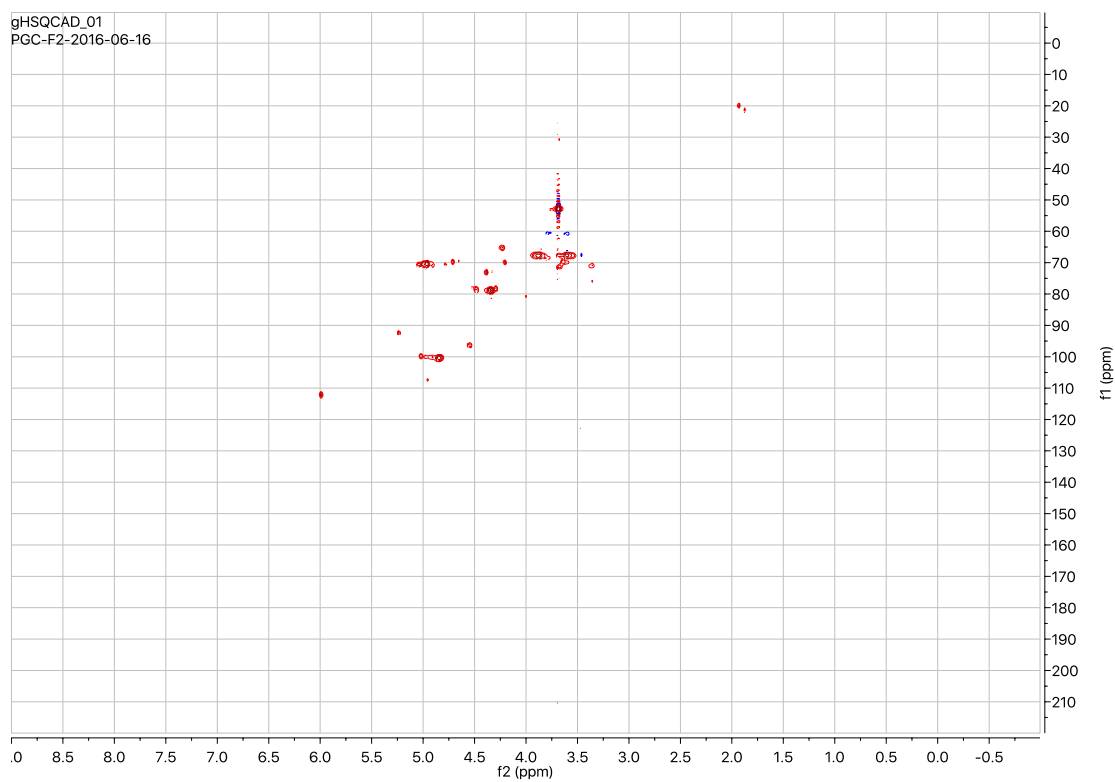


Figure S4: HSQC spectrum of 10% PGC.

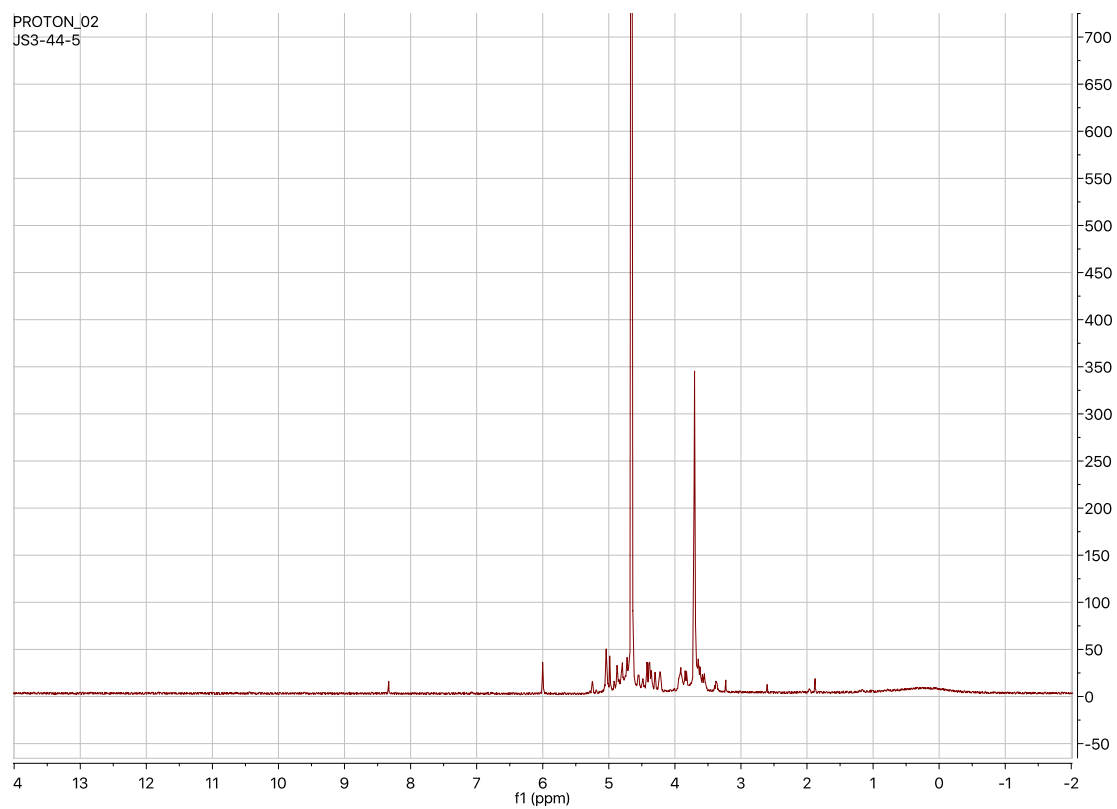


Figure S5: ^1H NMR spectrum of **4**.

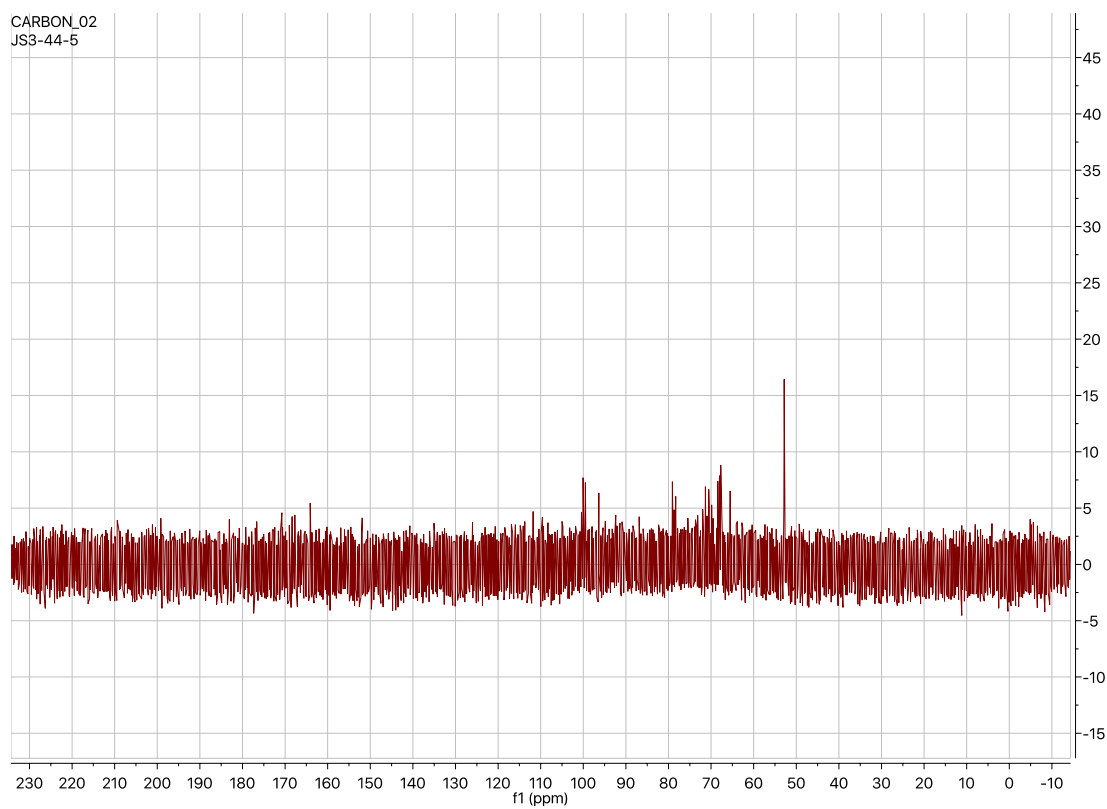


Figure S6: ^{13}C NMR spectrum of **4**.

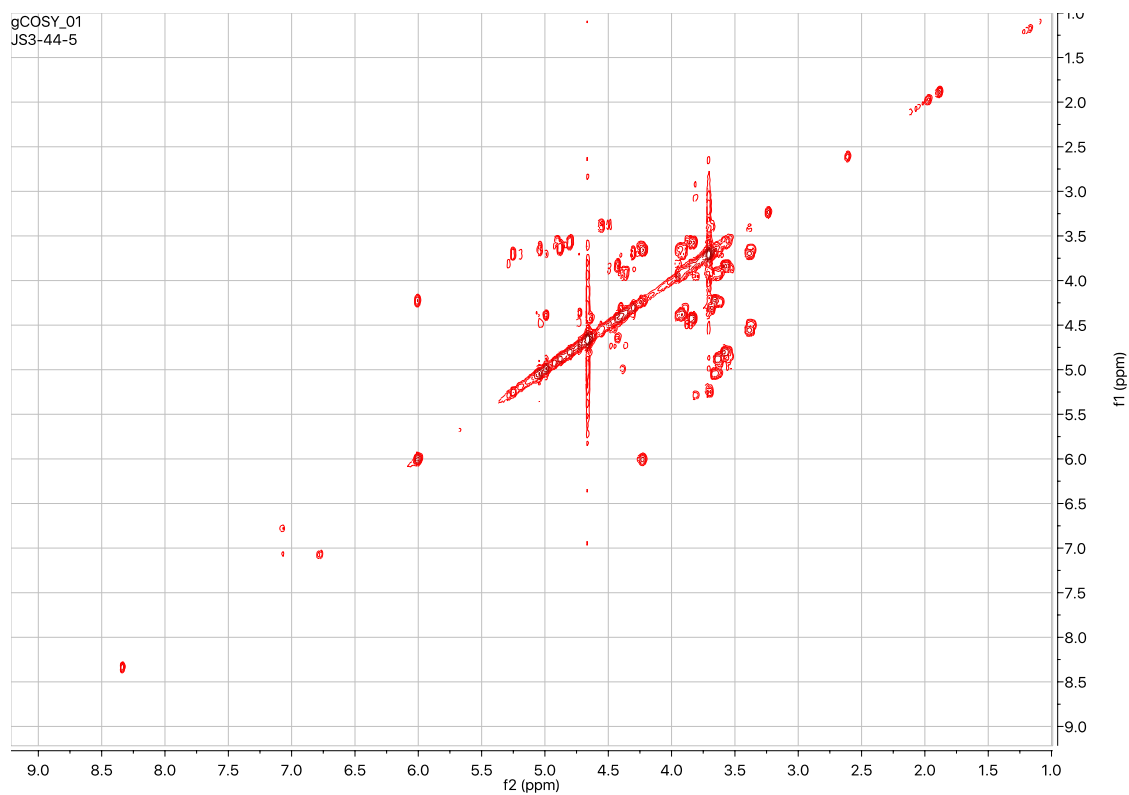


Figure S7: COSY spectrum of **4**.

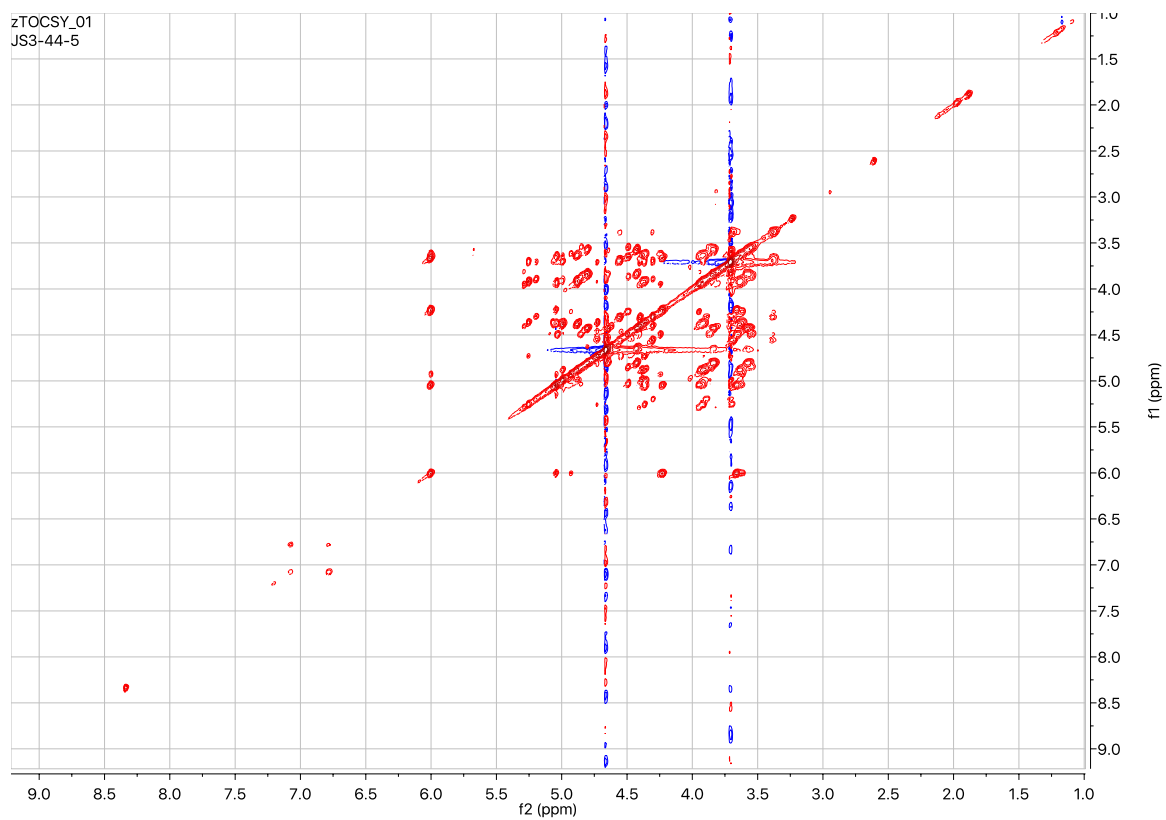


Figure S8: TOCSY spectrum of **4**.

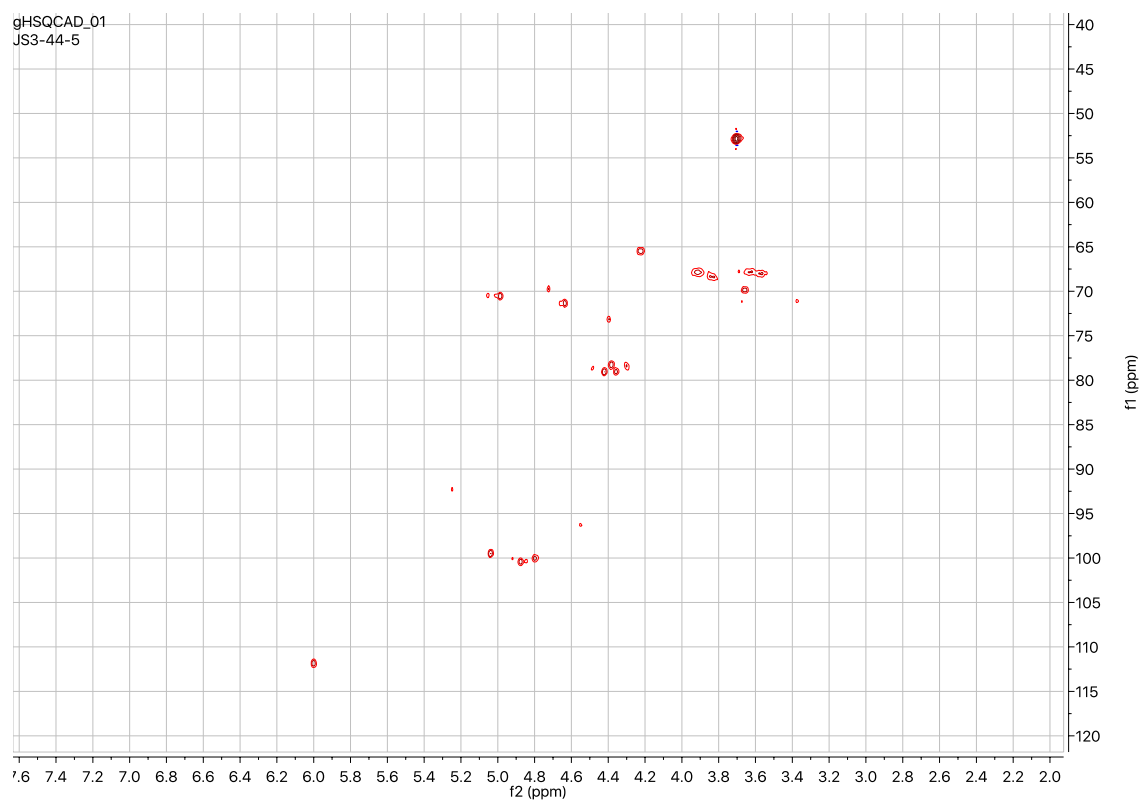


Figure S9: HSQC spectrum of **4**.

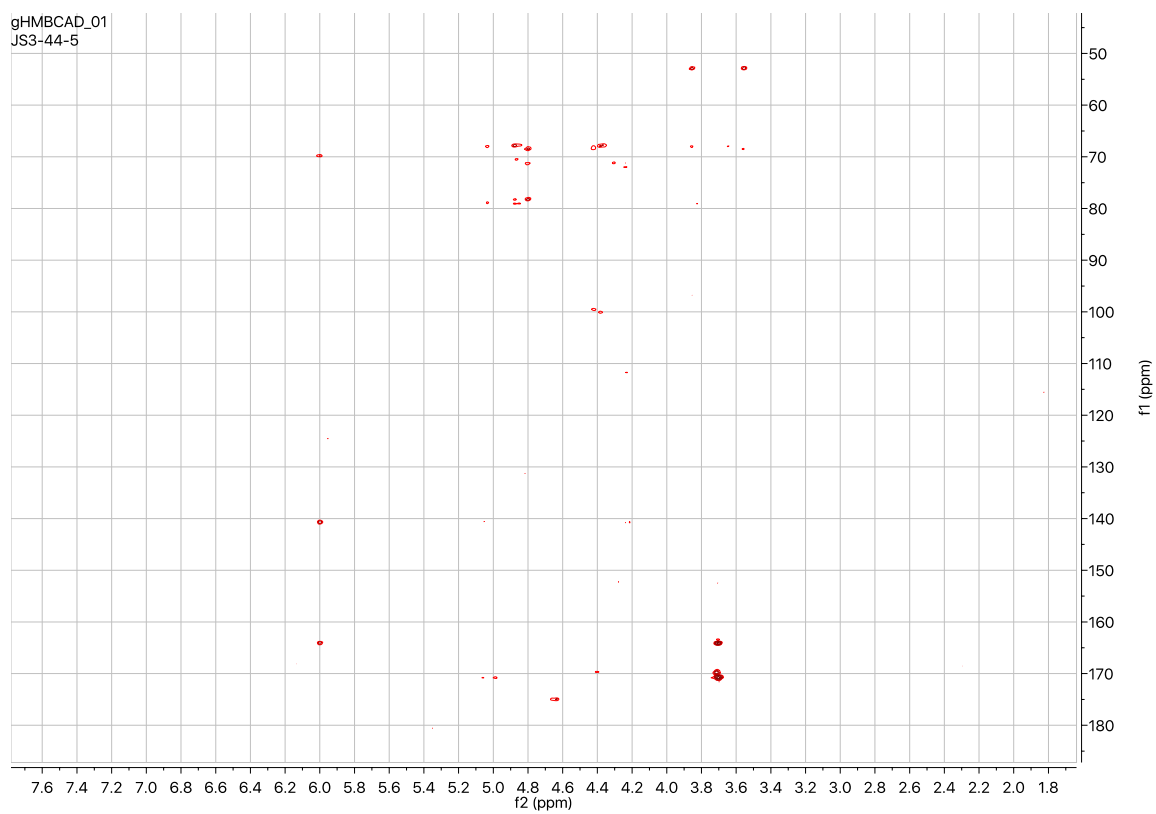


Figure S10: HMBC spectrum of **4**.

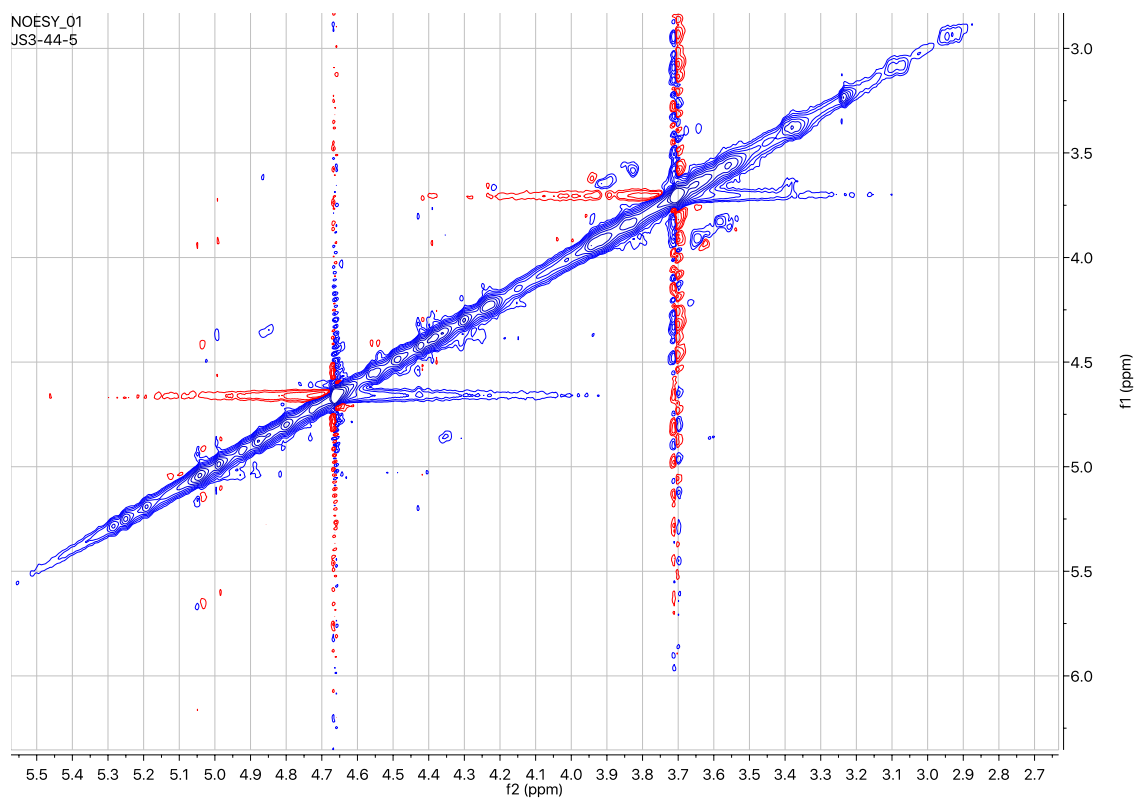


Figure S11: NOESY spectrum of **4**.

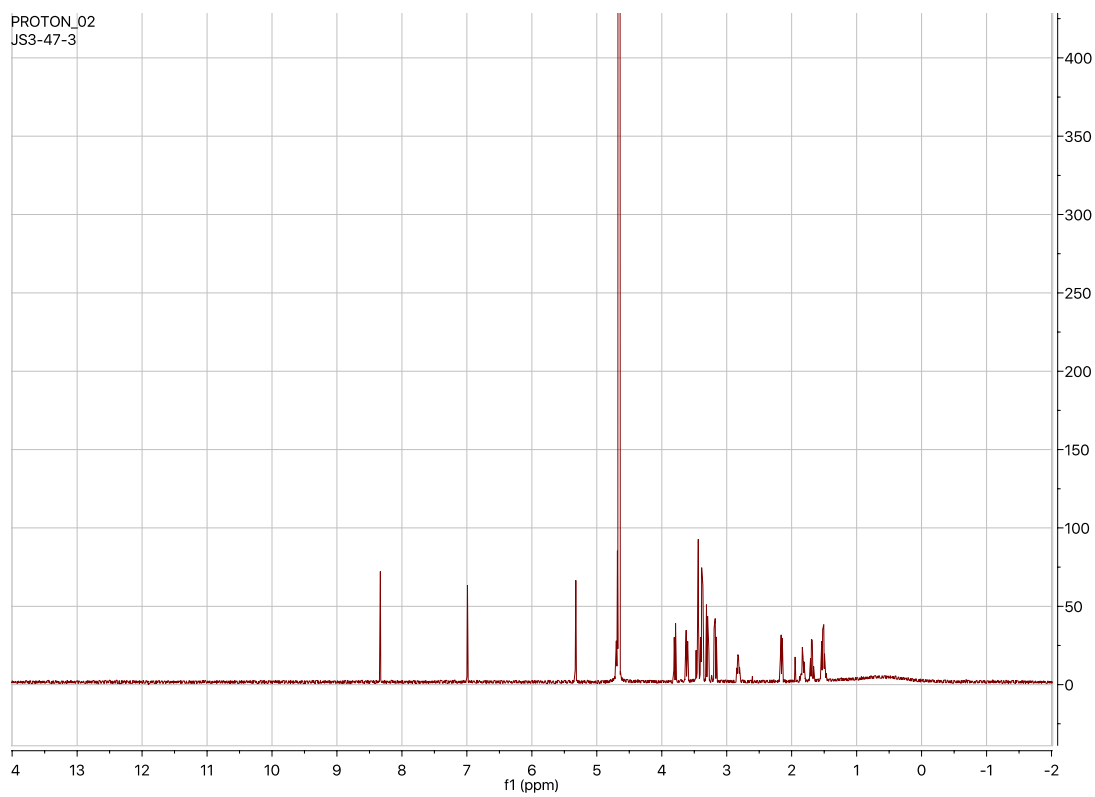


Figure S12: ^1H NMR spectrum of **1**.

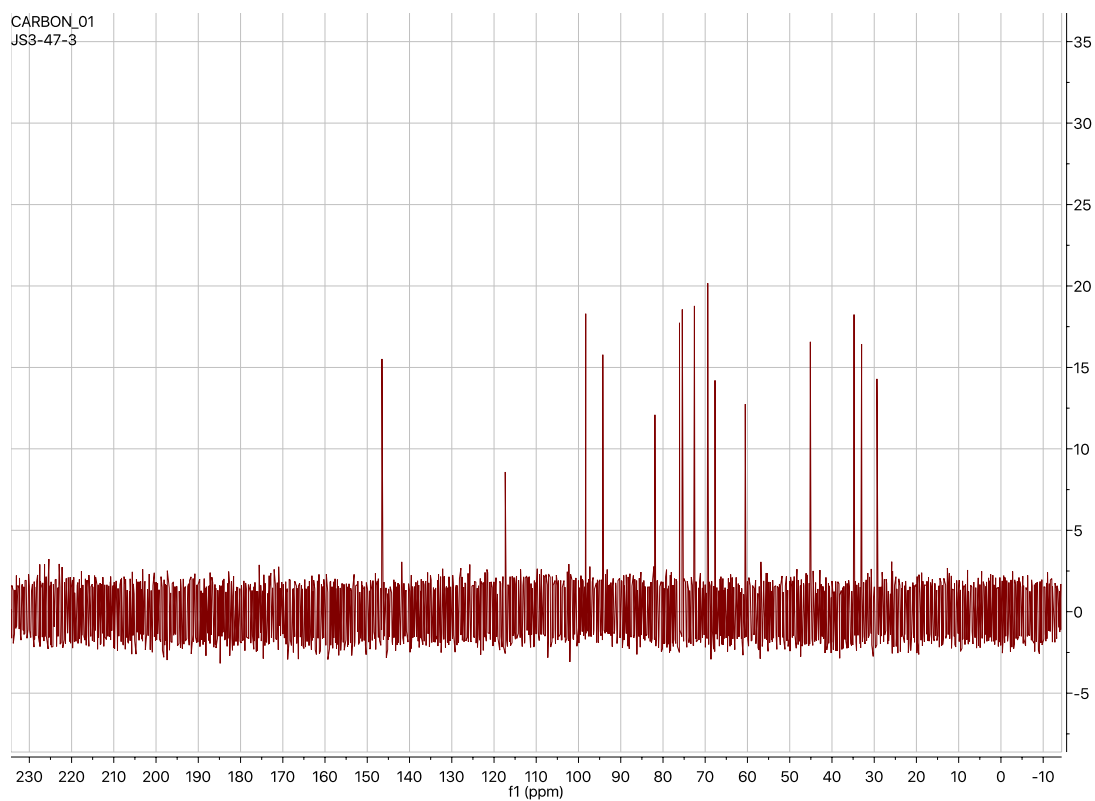


Figure S13: ^{13}C NMR spectrum of **1**.

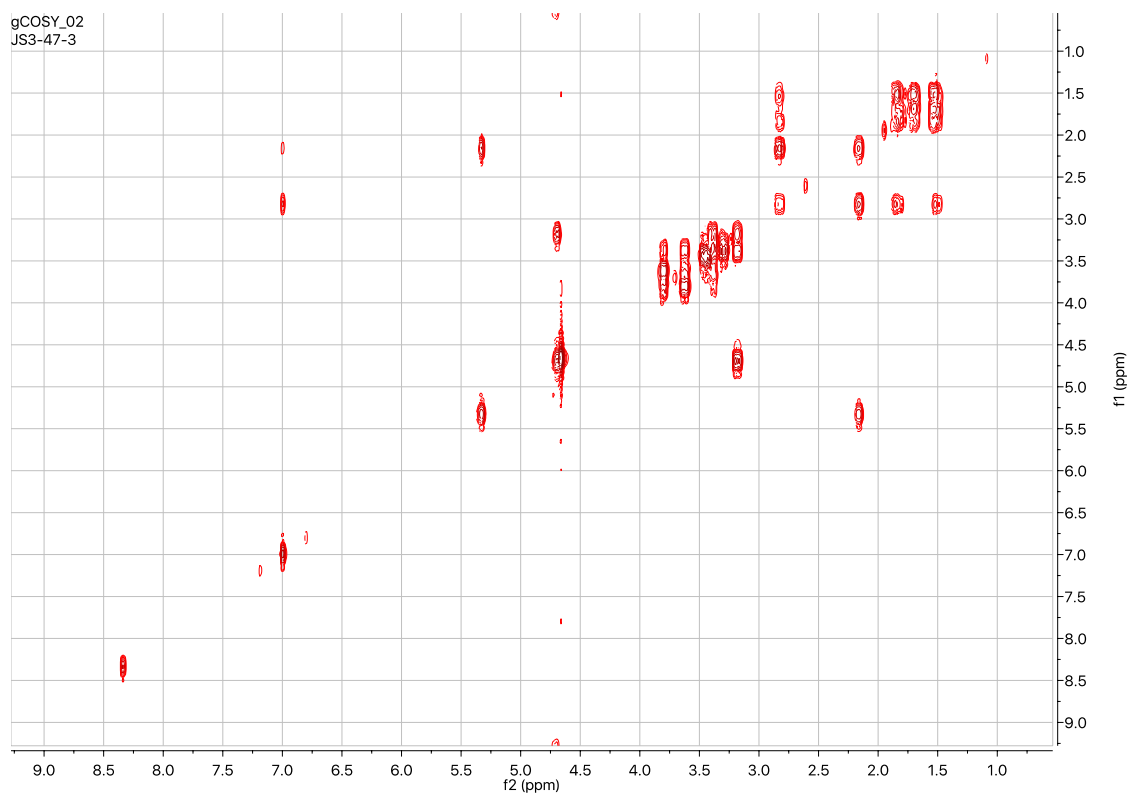


Figure S14: COSY spectrum of **1**.

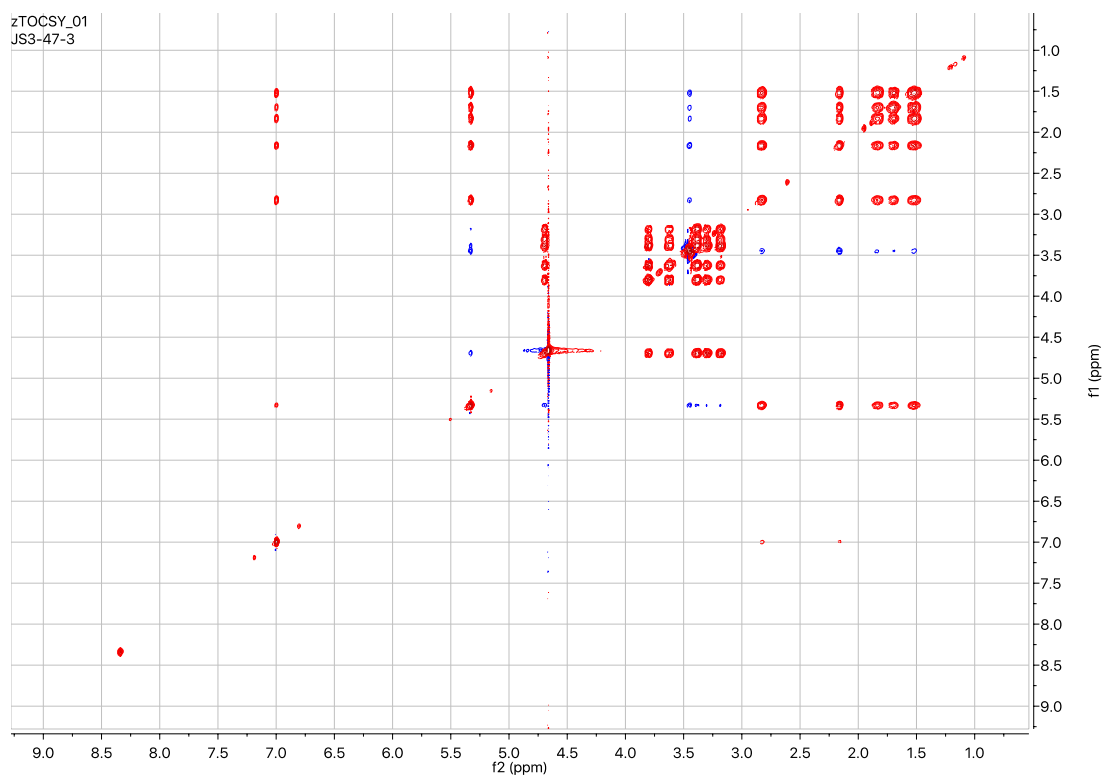


Figure S15: TOCSY spectrum of **1**.

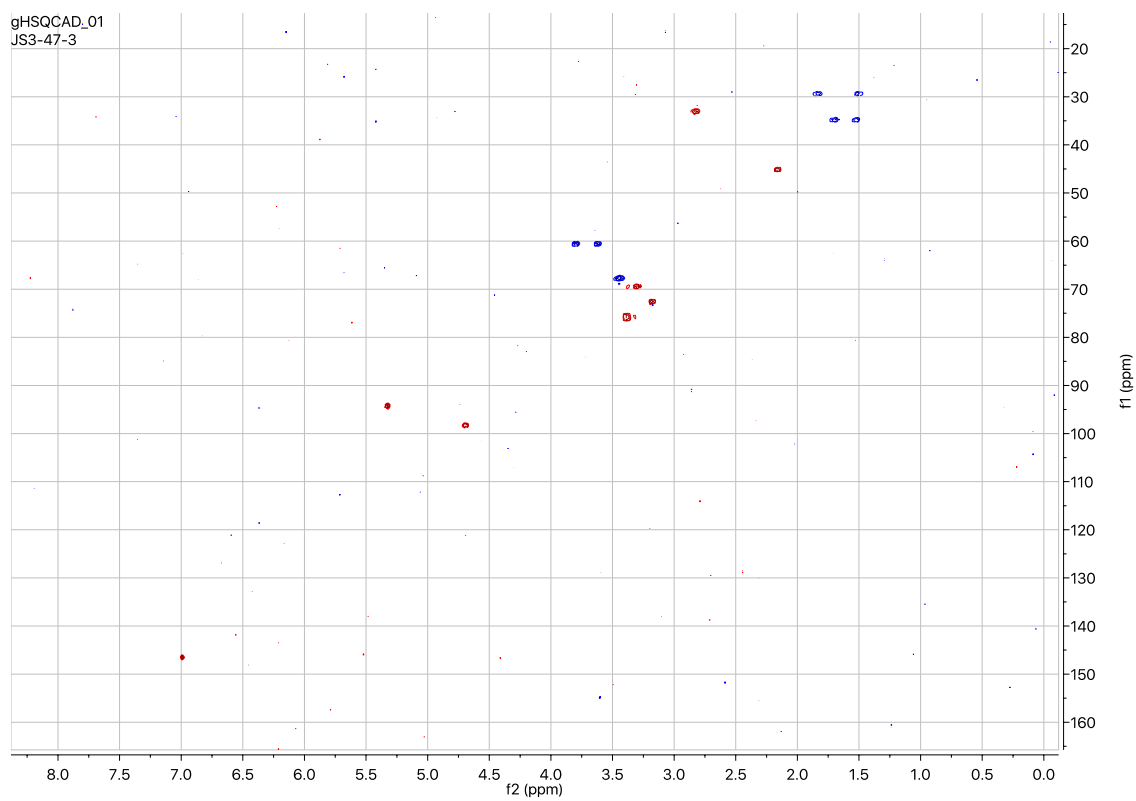


Figure S16: HSQC spectrum of **1**.

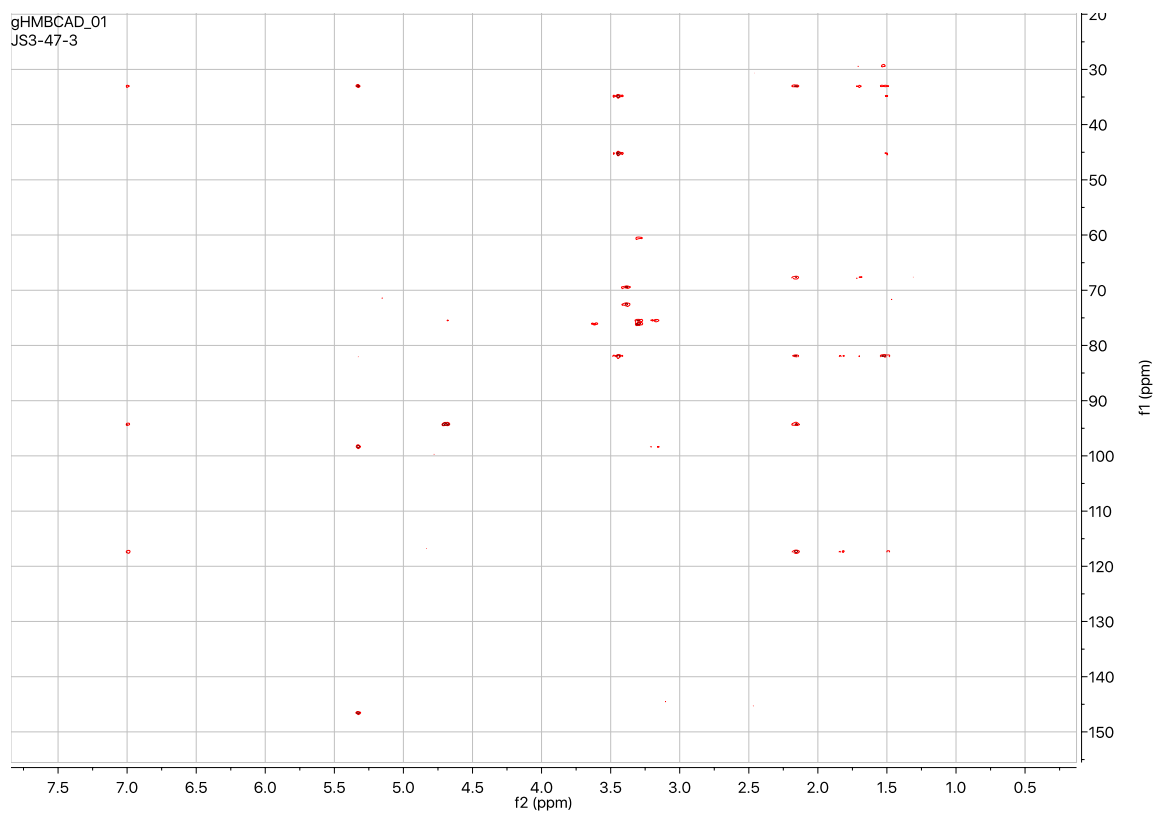


Figure S17: HMBC spectrum of **1**.

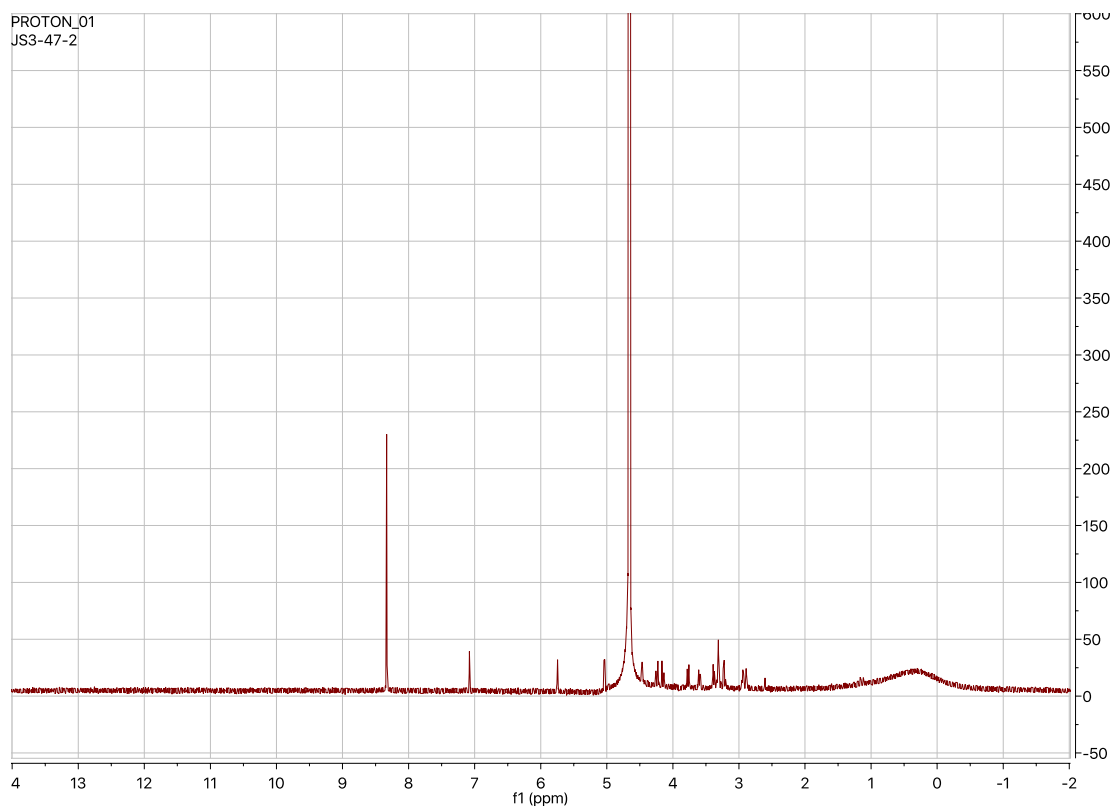


Figure S18: ^1H spectrum of **2**.

Figure S19: COSY spectrum of **2**.

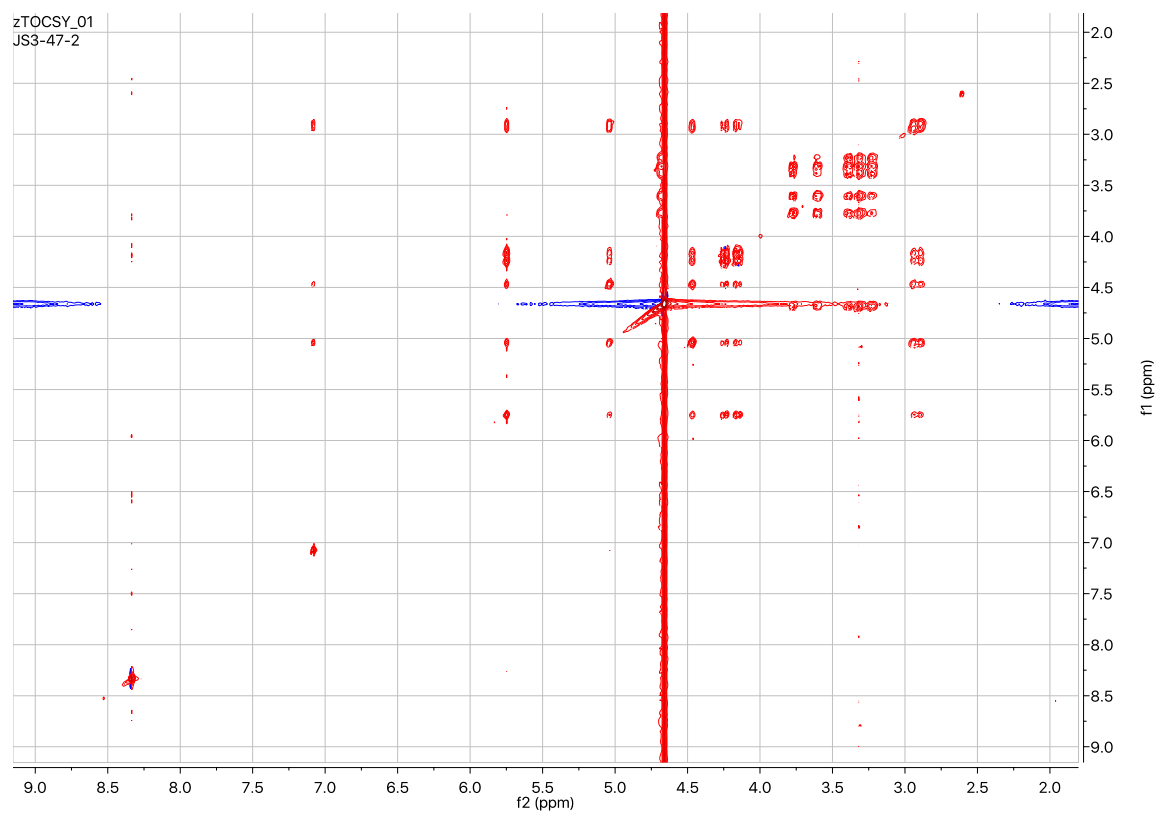


Figure S20: TOCSY spectrum of **2**.

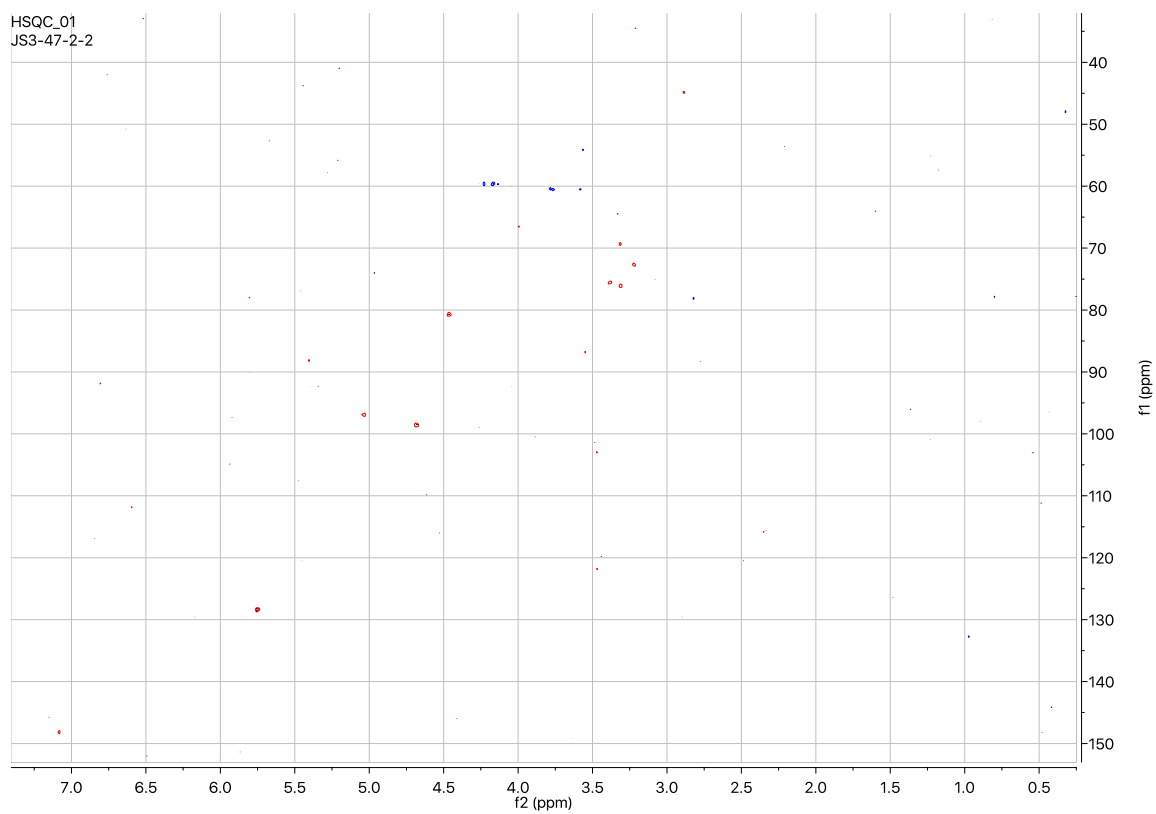


Figure S21: HSQC spectrum of **2**.

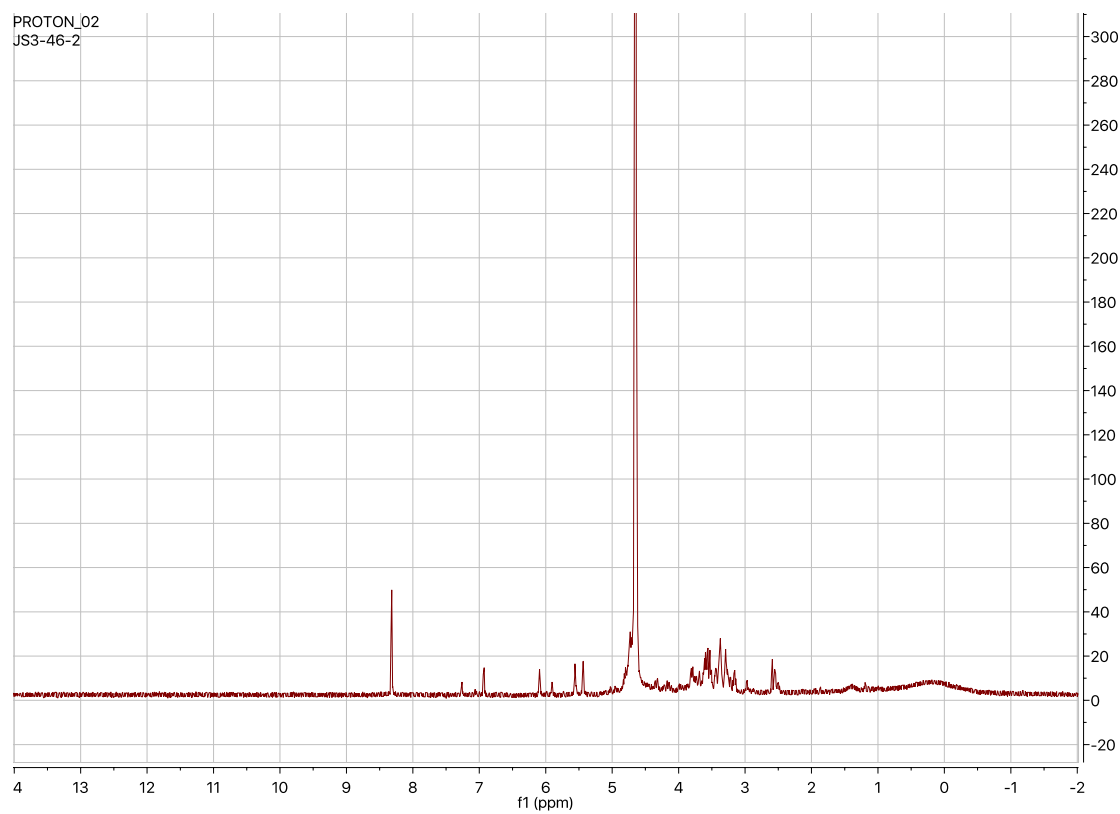


Figure S22: ^1H spectrum of **3**.

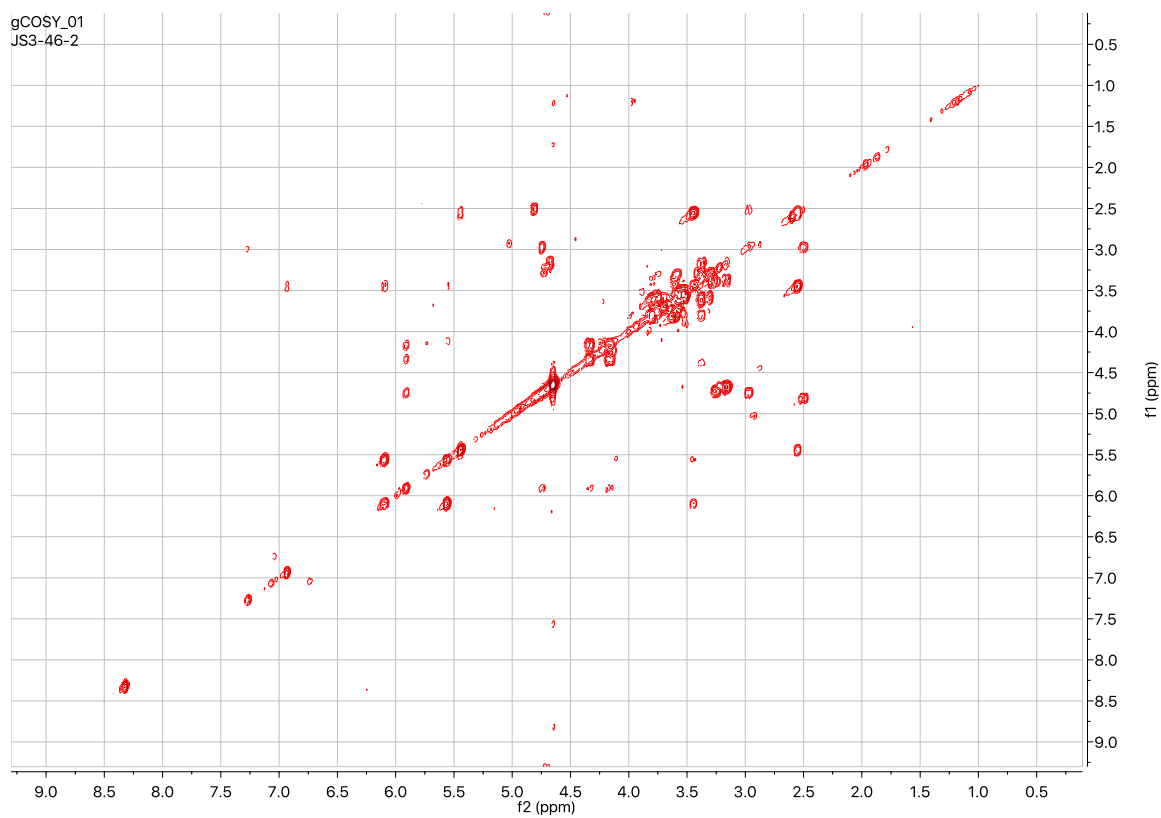


Figure S23: COSY spectrum of **3**.

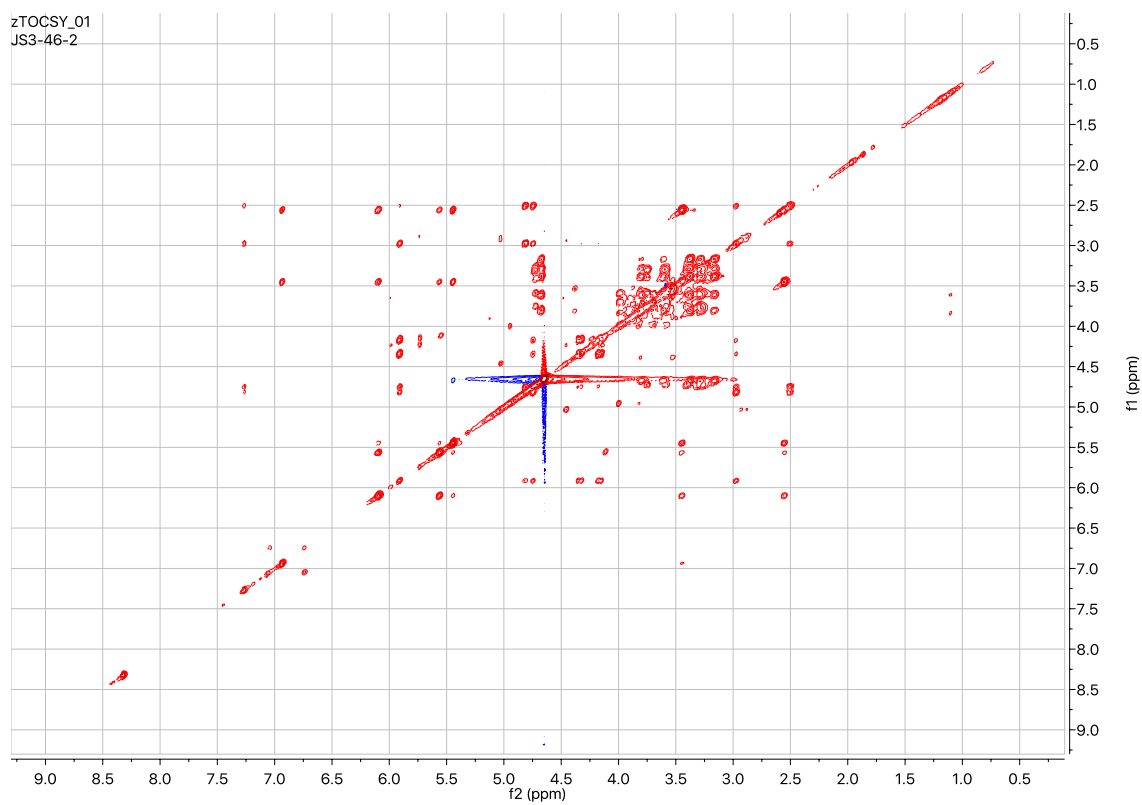


Figure S24: TCOSY spectrum of **3**.

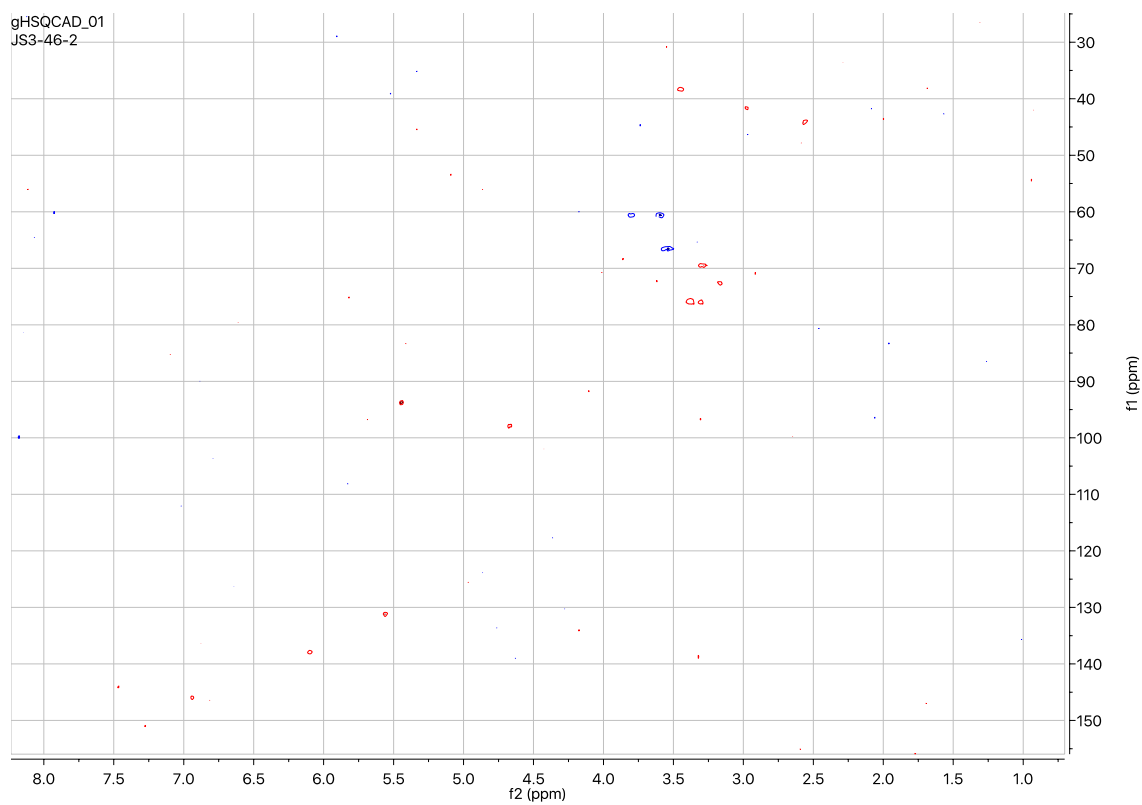


Figure S25: HSQC spectrum of **3**.

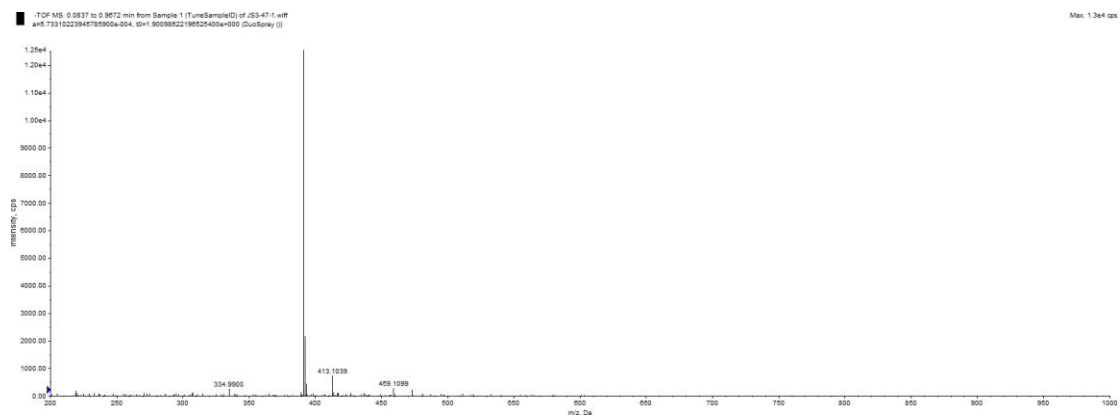


Figure S26: HR-MS of 1.

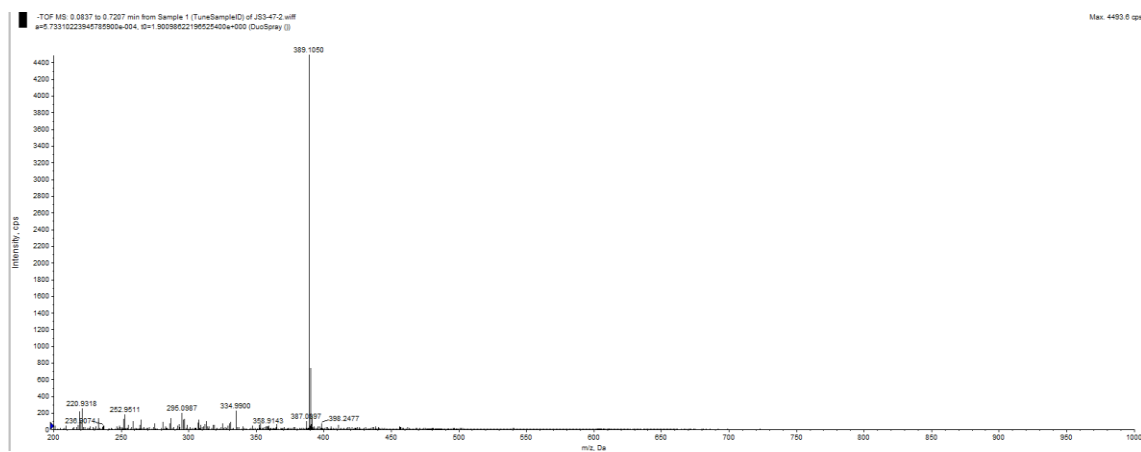


Figure S27: HR-MS of 2.

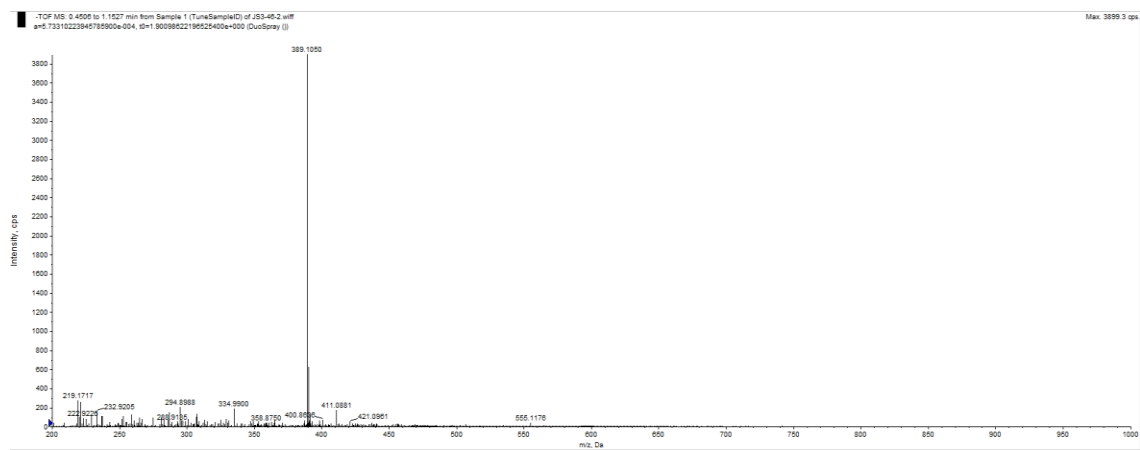


Figure S28: HR-MS of **3**.